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**14. ABSTRACT**

Hypertension and aneurysm are a prevalent problem in our society. Because the risk of these vascular diseases is too important to ignore, our studies are designed to provide exciting and original concepts to examine the etiologies of hypertension and aneurysm with regard to mechanosensory organelles (primary cilia). Especially within polycystic kidney disease, untreated hypertension can worsen kidney function. Aneurysm rupture in PKD patients also remains a devastating complication that often could result in stroke and death. Our studies therefore aim at investigating new ideas in understanding hypertension and aneurysm formation in PKD.

**15. SUBJECT TERMS**

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**Page**

## 1. INTRODUCTION:

Polycystic kidney disease (PKD) is characterized by formation of fluid-filled cysts in both kidneys. PKD patients will eventually have renal failure, with subsequent dialysis or renal transplant. The genes mutated in PKD include Pkd1 and Pkd2, encoded for polycystin-1 and polycystin-2, respectively. Many studies have shown that the baseline circulating NO is much lower in PKD patients. This suggests possible vascular dysfunction. The purpose of our research is to investigate cilia function in the vascular endothelial cells. More important, hypertension has been a critically important risk factor for cardiovascular diseases in PKD patients, which occur early in these individuals, compared to their age-matched cohorts, and still remains the most frequent cause of mortality even when their renal function is still normal. With our expertise and tools available in our laboratory, we thus hypothesize that cilia in the cilia play crucial and important roles in regulating PKD pathology. Specifically, we will provide the first insights into physiological functions and cellular pathways of primary cilia in vasculatures and in PKD.

## 2. KEYWORDS:

Cardiovascular, cilia, ciliopathy, ciliotherapy, endothelia, epithelia, polycystic kidney disease, polycystin-1, polycystin-2, primary cilia

## 3. ACCOMPLISHMENTS:

**Major Goals**

The main goal of the project is to determine the roles of primary cilia in PKD. This goal is divided into two major subaims, and our Statement of Work (SOW) is as follow.

Aim 1 (months 1-30). We will study mechanosensory function of endothelial cilia in hypertension.
Aim 1.1 (months 1-12): We will measure blood pressure in cilium mutant mice in vivo.
Aim 1.2 (months 13-30): We will examine signaling mechanisms of cilia & their effects on blood pressure.

Aim 2 (months 7-36). We will study mechanosensory function of endothelial cilia in vascular aneurysm.
Aim 2.1 (months 7-20): We will quantify aneurysm formation in cilium mutant mice in vivo.
Aim 2.2 (months 20-36): We will identify signaling mechanisms of cilia & their consequence on aneurysm.

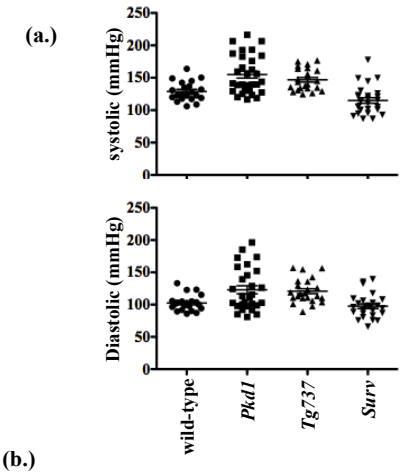
**Accomplishments**

<u>Aim 1.1 (months 1-12): measurement of blood pressure</u>

Over the past year, we have measured the systolic and diastolic blood pressure by non-invasive blood pressure system - tail cuff method with the aid of a computerized system (CODA system, Kent Scientific, Connecticut, USA). Measurements were performed at the baseline 3-times per week for 2 weeks after previous 3 days of training for each mouse. On each day of blood pressure measurement, 2 sets of 18 measurements were obtained including three measurements of training or acclimation. The measurements were averaged for each mouse with at least three mice for each genotype. All animals were tested by an investigator blinded to the genotypes of the animals. The data from the tail cuff method was also verified with limited studies with a more invasive, surgically implanted telemetry probe (data not shown).

When blood pressure was monitored in our mutant mice, *Survivin* knockout mice surprisingly did not show an elevated blood pressure (**Figure 1**). However, *Pkd1* and *Tg737* mice are hypertensive. Supporting this view, patients with PKD have a significantly greater chance to develop hypertension than general population.

**Figure 1**




(b.)

| | systolic (mmHg) | diastolic (mmHg) | N |
|---|---|---|---|
| wild-type | 128±9 | 102±7 | 3 |
| *Pdgfbcre:Pkd1*$^{flox/flox}$ | 150±19* | 120±21* | 4 |
| *Tie2Cre:Tg737*$^{flox/flox}$ | 147±10* | 120±11* | 3 |
| *Pdgfβcre:Surv*$^{flox/flox}$ | 114±13 | 99±13 | 3 |
| ** significant different from wild-type control* | | | |

**Figure 1.** Blood pressure was measured in wild-type and conditional *Pkd1*, *Tg737*, and *Survivin* (*Sur*) mice for a 2-week period (***a***). The averaged measurements of blood pressure during this 2-week period were tabulated (***b***).

**Figure 2**

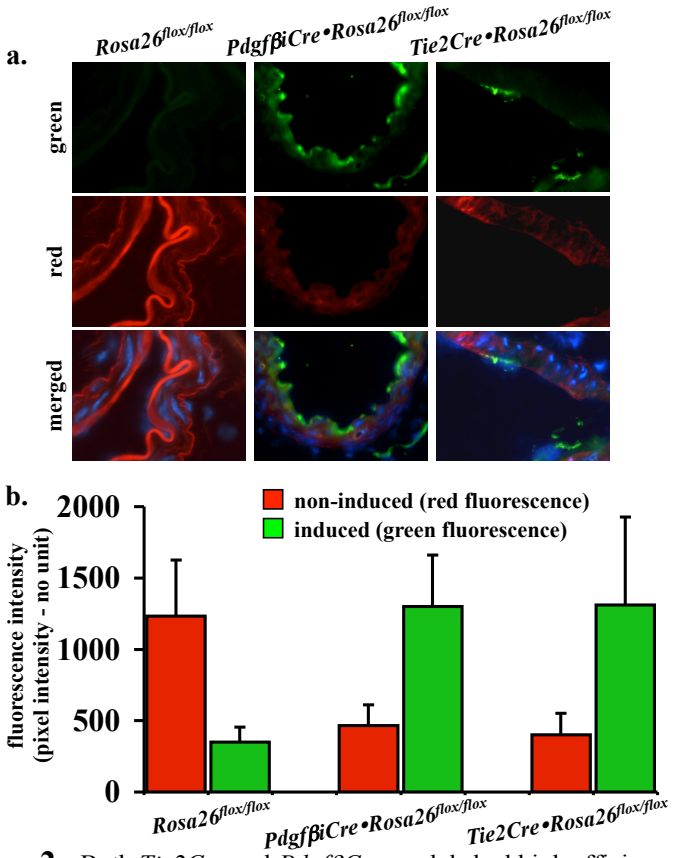



**Figure 2.** Both *Tie2Cre* and *PdgfβCre* models had high efficiency in gene deletion to turn vascular endothelia from red to green fluorescence.

Aim 2.1 (months 7-20): quantify aneurysm formation

We have started Aim 2.1 in the last couple of months. Our animal use for these studies has been approved by our Institutional Animal Care and Use Committee. We will use both traditional and conditional transgenic mouse models in our present studies. We have previously obtained traditional transgenic *Pkd*-mouse models *Pkd2*$^{+/-}$ and *Tg737*$^{Orpk/Orpk}$. We will also use *survivin*$^{flox/flox}$ and *Pkd1*$^{flox/flox}$ conditional mouse models. To generate kidney-specific knockout, we have bred our mice with *Mx1Cre* mice. To generate vascular-specific knockout, we have used and confirmed *PdgfβCre* and *Tie2Cre* mice (**Figure 2**). Briefly, one-week old pups were injected intra-peritoneally with 62.5 μg of 50 μL polyinosinic:polycytidylic ribonuceic acid (pI:pC) every day for five consecutive days.

*Tie2Cre* or *PdgfβCre* mouse was bred with *Gt(ROSA)26Sor* (*Rosa26*) mouse, resulting in either *PdgfβCre•Rosa26* or *Tie2Cre•Rosa26* genotype. The *Rosa26* mouse was used as a control. These 3 mouse groups were induced to activate Cre which acts on *Rosa26* allele. The *Rosa26* genetic background is used as a

reporter system to verify and validate the efficiency of the *Cre* mice. The *Rosa26* includes its inducible fluorescence reporter system; i.e. all endothelia lining the vasculatures have red fluorescence (non-induced). The red fluorescence will be replaced with green fluorescence upon *Cre* recombinant (induction). Abdominal aortas were isolated, stained with nuclear marker (blue), and imaged for their green/red fluorescence (**Figure 2a**). Quantitation analysis of vascular-lining endothelia indicates high efficacy of both *PdgfbCre* and *Tie2Cre* backgrounds to delete a specific gene in vascular lining endothelia (**Figure 2b**). N=4 for each genotype and treatment.

**Training and Professional Development**

The project will continue to be part of our research-training program for two postdoctoral fellows and one graduate student. All of our trainees will continue working on the proposed studies, and results of their studies will be presented in conferences, such as FASEB meeting. This is an important meeting for our trainees to demonstrate their research productivity. We meet one on one in my office regularly to discuss any technical problems that may have arisen from their experiments. All trainees also participate in our laboratory meeting. All are expected to present their research progress in our laboratory meeting.

In addition, the Postdoctoral Research Committee and Graduate Committee will also evaluate the productivity of our postdoctoral fellows and graduate student, respectively. For most of the technical skills and methods, they will be trained within our laboratory and our surrounding laboratories. For more specialized skills, we will encourage them to attend various workshops. All trainees are recommended and student is required to the following courses.

- PHS601- Research Ethics and Regulations
- PHS 614- Biologics
- PHS 636- Proteomics

**Results Dissemination**

No outreach activity was attempted. Relevant results have been published and deposited into the National Library of Medicine.


1. Liu T, Jin X, Prasad RM, Sari Y, **Nauli SM**. Three types of ependymal cells with intracellular calcium oscillation are characterized by distinct cilia beating properties. J Neurosci Res. 2014 Sep;92(9):1199-204. [PMID:24811319]
2. Muntean BS, Jin X, Williams FE, **Nauli SM**. Primary cilium regulates CaV1.2 expression through Wnt signaling. J Cell Physiol. 2014 Dec;229(12):1926-34. [PMID:24700505]
3. Atkinson KF, Kathem SH, Jin X, Muntean BS, Abou-Alaiwi WA, Nauli AM, **Nauli SM**. Dopaminergic signaling within the primary cilia in the renovascular system. Front Physiol. 2015 Apr 16;6:103. [PMID:25932013]
4. Mohieldin AM, Zubayer HS, Al Omran AJ, Saternos HC, Zarban A, **Nauli SM**, AbouAlaiwi WA. Vascular Endothelial Primary Cilia: Mechanosensation and Hypertension. Curr Hypertens Rev. 2015 Jun 30 [In Press; PMID:26122329]


**Future Goal**

Our goal for next funding year is to complete Aim 2.1 and to initiate Aim 1.2.

## 4. IMPACT:

### Impact on the discipline

Primary cilia are sensory organelles that extend from the cell surface and sense extracellular signals. Endothelial primary cilia protruding from the inner surface of blood vessel walls sense changes in blood flow and convert this mechanosensation into an intracellular biochemical/molecular signal, which triggers a cellular response. Endothelial cilia dysfunction may contribute to the impairment of this response and thus be directly implicated in the development of vascular abnormalities such as hypertension as seen in our PKD animal models. The completion of this project may present primary cilia as a novel therapeutic target for vascular hypertension.

### Impact on other disciplines

Although our project is directed to lower blood pressure in patients with PKD, it may apply to a general population with hypertension. The completion of our project may serve as a proof of comcept for targeted-clinical therapy on primary cilia as a novel mechanism in general hypertensive patients.

### Impact on technology transfer

Nothing to Report at this time.

### Impact on society

Nothing to Report.

## 5. CHANGES AND PROBLEMS:

Nothing to Report.

## 6. PRODUCTS:


1. Liu T, Jin X, Prasad RM, Sari Y, **Nauli SM**. Three types of ependymal cells with intracellular calcium oscillation are characterized by distinct cilia beating properties. J Neurosci Res. 2014 Sep;92(9):1199-204. [PMID:24811319]
2. Muntean BS, Jin X, Williams FE, **Nauli SM**. Primary cilium regulates CaV1.2 expression through Wnt signaling. J Cell Physiol. 2014 Dec;229(12):1926-34. [PMID:24700505]
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4. Mohieldin AM, Zubayer HS, Al Omran AJ, Saternos HC, Zarban A, **Nauli SM**, AbouAlaiwi WA. Vascular Endothelial Primary Cilia: Mechanosensation and Hypertension. Curr Hypertens Rev. 2015 Jun 30 [In Press; PMID:26122329]

## 7. PARTICIPANTS:

### Individuals

| | |
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| Nearest person month worked: | *~9* |
| Contribution to Project: | *Dr. Atkinson has been working on the in vivo mouse model.* |
| Funding Support: | *This award* |

| | |
|---|---|
| Name: | *Pala Rajasekharreddy* |
| Project Role: | *Postdoctoral Fellow* |
| Researcher Identifier (e.g. ORCID ID): | *N/A* |
| Nearest person month worked: | *~6* |
| Contribution to Project: | *Dr. Rajasekharreddy has been working on the in vitro assay.* |
| Funding Support: | *This award* |

| | |
|---|---|
| Name: | *Rinzhin Sherpa* |
| Project Role: | *Graduate Student* |
| Researcher Identifier (e.g. ORCID ID): | *N/A* |
| Nearest person month worked: | *~9* |
| Contribution to Project: | *Mr. Sherpa has been working on the in vivo mouse model and in vitro cilia measurement.* |
| Funding Support: | *This award* |

### Change in Other Supports or Key Personnel

Nothing to Report

### Partner Organization

Nothing to Report

## 8. SPECIAL REPORTING REQUIREMENTS:

Not applicable

## 9. APPENDICES:

See Below

Journal of Neuroscience Research 92:1199–1204 (2014)

# Three Types of Ependymal Cells With Intracellular Calcium Oscillation Are Characterized by Distinct Cilia Beating Properties

**Tongyu Liu, Xingjian Jin, Rahul M. Prasad, Youssef Sari, and Surya M. Nauli***

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Ependymal cells are multiciliated epithelial cells that line the ventricles in the adult brain. Abnormal function or structure of ependymal cilia has been associated with various neurological deficits. For the first time, we report three distinct ependymal cell types, I, II, and III, based on their unique ciliary beating frequency and beating angle. These ependymal cells have specific localizations within the third ventricle of the mouse brain. Furthermore, neither ependymal cell types nor their localizations are altered by aging. Our high-speed fluorescence imaging analysis reveals that these ependymal cells have an intracellular pacing calcium oscillation property. Our study further shows that alcohol can significantly repress the amplitude of calcium oscillation and the frequency of ciliary beating, resulting in an overall decrease in volume replacement by the cilia. Furthermore, the pharmacological agent cilostazol could differentially increase cilia beating frequency in type II, but not in type I or type III, ependymal cells. In summary, we provide the first evidence of three distinct types of ependymal cells with calcium oscillation properties. 

**Key words:** cilia; cerebrospinal fluid; calcium

Cilia are generally classified as solitary nonmotile and bundled motile organelles (Abou Alaiwi et al., 2009a; Nauli et al., 2011). Motile and nonmotile cilia have been implicated in fundamental processes of development and disease. Motile cilia can be found in the ependymal cells, forming a lining in the brain ventricles and central canal of the spinal cord. Ependymal cells are ciliated, simple cuboidal, epithelium-like glial cells that move cerebrospinal fluid (CSF) along the ventricles (Del Bigio, 1995). Abnormal ependymal cilia result in hydrocephalus induced by anomalous flow of CSF (Banizs et al., 2005; Baas et al., 2006; Wodarczyk et al., 2009; Tissir et al., 2010).

Ependymal cells also play an important role in regulating pluripotent neural stem cells (Rietze et al., 2001). Beating of ependymal cilia is required for normal CSF flow, which functions as a guide for specific directional migration of new neurons (Sawamoto et al., 2006). The coupling between ependymal cilia beating and hydrodynamic forces has been proposed to regulate planar cell polarity during development or stroke (Guirao et al., 2010; Mirzadeh et al., 2010; Devaraju et al., 2013). In addition, ependymal cilia play major roles in CSF dynamics, cerebral fluid balance, secretion, toxin metabolism, and many other functions (Genzen et al., 2009; Appelbe et al., 2013). Although ependymal cells regulate CSF flow, which regulates many neuronal processes, different types of ependymal cells have not been distinguished. We show here, based on the cilia beating frequency and beating angle, that ependymal cells can be distinctly categorized into three types. Furthermore, each type of ependymal cell is uniquely localized within the ventricle.

## MATERIALS AND METHODS

All animal experiments were approved by the University of Toledo's Institutional Animal Care and Use Committee (IACUC). The wild-type mice were euthanized with carbon dioxide for 5 min. After craniotomy, the whole brain was removed. The sagittal slice was dissected with a thickness of about 100 μm and was immediately embedded in Dulbecco's modified Eagle's medium (DMEM; Cellgro Corning Life Sciences-Mediatech, Manassas, VA) at 39°C in the presence of 95%/5% $O_2/CO_2$ mixture.

### Immunofluorescence Microscopy

The brain slice was fixed in phosphate buffer containing 3% paraformaldehyde and 2% sucrose for 10 min. Mouse primary antibody antiacetylated α-tubulin was used at a dilution of 1:5,000 (Sigma, St. Louis, MO). The brain slice was

Additional Supporting Information may be found in the online version of this article.

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incubated in primary antibody solution overnight. Secondary antibody fluorescein anti-mouse IgG was used at a dilution of 1:500 (Vector Laboratories, Burlingame, CA), and the brain slice was incubated with secondary antibody solution for 1 hr. Before observation under a TiU fluorescent microscopy (Nikon, Tokyo, Japan), the section was dyed with DAPI for 5 min (Abou Alaiwi et al., 2014).

### Measurement of Cilia Beating Frequency

The prepared brain slice was kept in a customized glass-bottom plate covered with 500 μl DMEM containing 2% B27 at 39°C. In some cases, 0.25% ethanol was added to the media. The video of cilia beating was captured with a TiU high-resolution differentiation interference contrast microscope. The capture rate of the video was 5 msec for a minimum of 1 sec (200 frames per sec).

### Measurement of Fluid Movement and Volume Replacement

Because of the transparency of the buffer solution, we used 200-nm latex beads (Molecular Probes, Eugene, OR) to help analyze speed in the solution movement. The velocity of fluid movement was calculated by tracing one single nanobead flowing across the third ventricle wall. The overall fluid volume moved by ependymal cilia was calculated with the following formula: volume replacement ($\mu m^3$/stroke) = fluid movement velocity ($\mu m^3$/sec)/cilia beating frequency (stroke/sec).

### Calcium Signal Recording

To record cytosolic calcium oscillation, the brain slice was incubated with 20 μg/ml fluo-2 (TEFLabs, Austin, TX) for 30 min at 39°C. The tissue was then transferred to a glass-bottom plate covered with 500 μl DMEM containing 2% B27 (Gibco, Rockville, MD) at 39°C. In some cases, 0.25% ethanol was added to the media. The video of calcium oscillation was recorded at a capture rate of 5 msec for a minimum of 1 sec (200 frames per sec), with excitation and emission wavelengths of 488 nm and 515 nm, respectively.

## RESULTS

### Ependymal Cells Can Be Classified Into Three Types Based on Their Cilia Beating Frequency

We cut the mouse brain in a sagittal plane to enhance our observation of the entire third ventricle. To verify our high-resolution differential interference contrast and fluorescence microscope systems, we examined the presence of ependymal cilia in the third ventricle (Fig. 1). Ependymal cilia were confirmed with a ciliary marker, acetylated-α-tubulin. Although in the control permeabilized brain no fluid movement was observed in nonbeating ependymal cilia (Supp. Info. Movie 1), we could observe the direction of fluid movement via oil ink in a freshly prepared brain ex vivo (Supp. Info. Movie 2).

We next attempted to quantify the cilia beating frequency. Based on our observation of individual ependymal cells from 87 independent experiments, we were surprised to notice that there were wide variations in the beating frequencies of ependymal cilia (Fig. 2). We could accurately assign each ependymal cell to one of three classifications depending on its ciliary beating. Type I ependymal cells

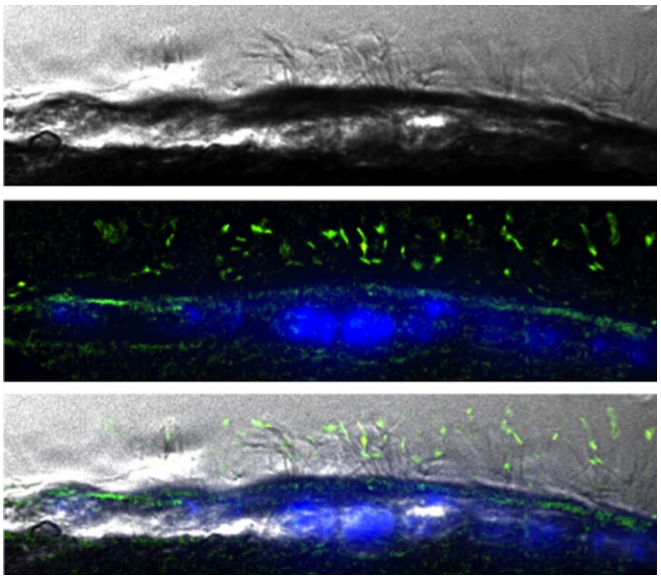

Fig. 1. The presence of ependymal cilia in mouse brain. Shown here are ependymal cells from the third ventricle of a mouse brain. The brain section was stained with anti-acetylated-α-tubulin, a ciliary marker (green), and counterstained with DAPI, a nucleus marker (blue). Individual differential interference contrast (**top**), fluorescence (**middle**), and merged (**bottom**) images are shown. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

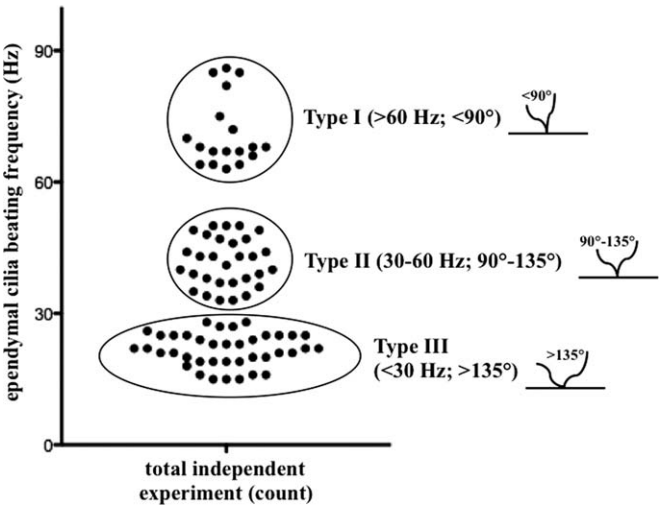


Fig. 2. Categories of ependymal cells in mouse brain. Based on a total of 87 independent experiments or preparations (represented as dots), ependymal cells could be classified into three types (grouped within circle or oval lines). Type I ependymal cilia had the highest beating frequency (>60 Hz), with a ciliary beating angle of less than 90°. Type II ependymal cilia had a medium beating frequency (30–60 Hz), with a ciliary beating angle between 90° and 135°. Type III ependymal cilia had the slowest beating frequency (<30Hz), with a ciliary beating angle of greater than 135°. The cartoon on the right depicts the beating angles of ependymal cilia.

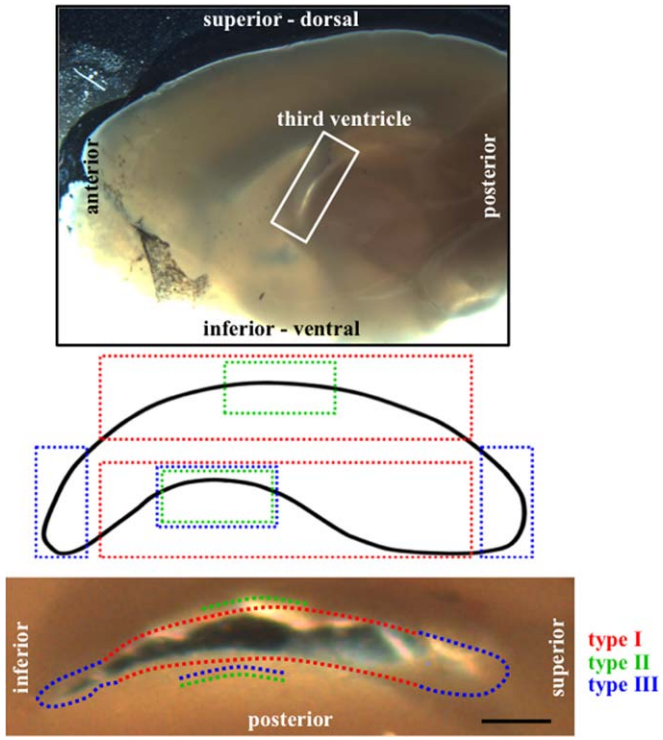


Fig. 3. Differential ependymal cell types and localization. Based on the ependymal cilia beating frequency, different ependymal cell types were enriched at certain locations within the third ventricle. A freshly cut brain section was imaged to show the third ventricle region (**top**). The cartoon (**middle**) and brain section (**bottom**) of the third ventricle depict localizations of different ependymal cells from a sagittal view. Scale bar = 30 μm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

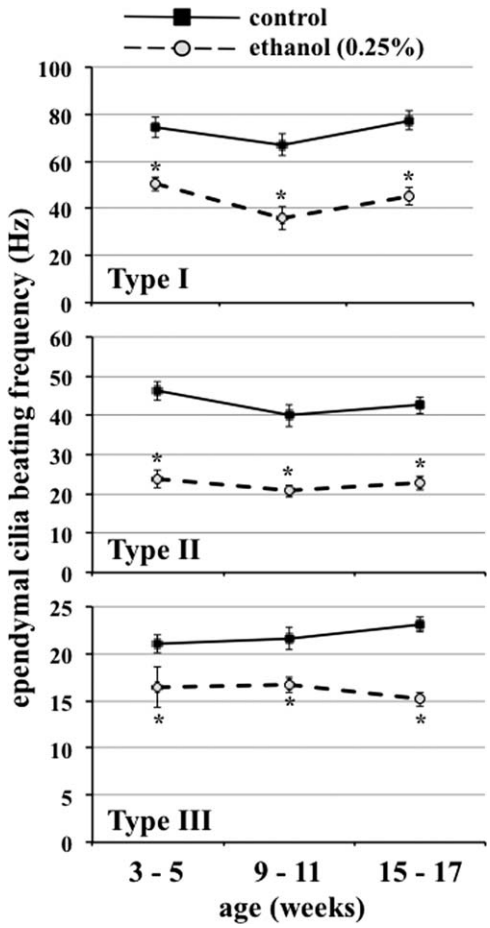


Fig. 4. Ependymal cilia beating frequency in different mouse age groups. All types of ependymal cilia in control and alcohol-treated mouse third ventricles were studied at different ages. Although beat frequency of ependymal cilia was not affected by age, acute alcohol treatment significantly decreased cilia beating frequency in all types of ependymal cells. At least three independent preparations were used for each ependymal cell type and age group.

had the highest beating frequency (>60Hz) and had a beating angle of less than 90° (Supp. Info. Movie 3). Type II ependymal cells had a medium beating frequency (30–60 Hz) and had a beating angle between 90° and 135° (Supp. Info. Movie 4). Type III ependymal cells had the slowest beating frequency (<30 Hz) and had a beating angle of more than 135° (Supp. Info. Movie 5).

To understand the difference in localizations among these ependymal cells, we mapped the distributions of ependymal cell types within the third ventricle (Fig. 3). Our mapping analysis indicated that type I cells were widely distributed along the ventricle walls, but they were absent at both inferior and superior corners of the third ventricle. Type II cells were observed mainly on the anterior wall of the ventricle, but they could also be found at the posterior wall of the ventricle. Type III cells were distributed almost exclusively in the inferior and superior corners of the third ventricle. Of note is that we could find all three cell types at the lower wall of the third ventricle.

## Cilia Beating Frequency Is Age Independent in Third Ventricles

Because ependymal cilia have been proposed to move CSF to support migration of various substances (Banizs et al., 2005; Sawamoto et al., 2006), we next sought to understand the role of aging in ependymal cilia beating. Mice were grouped according to age: 3–5 weeks, 9–11 weeks, and 15–17 weeks. The data indicate that we could distinctively classify the ependymal cilia beating into three types regardless of the age groups (Fig. 4). More importantly, there was no evidence that age was a factor in regulating ependymal cilia beating.

## Ependymal Cilia Beating Can Be Repressed by Ethanol

To confirm further that our classification of ependymal cells was valid and consistent, we preformed chemical screening on the cilia beating frequency. Ethanol at a concentration of 0.25% provided us with the most consistent changes in ependymal cilia beating. The data indicated that ethanol repressed ependymal cilia beating regardless of the age group (Fig. 4). Most importantly, ethanol repressed

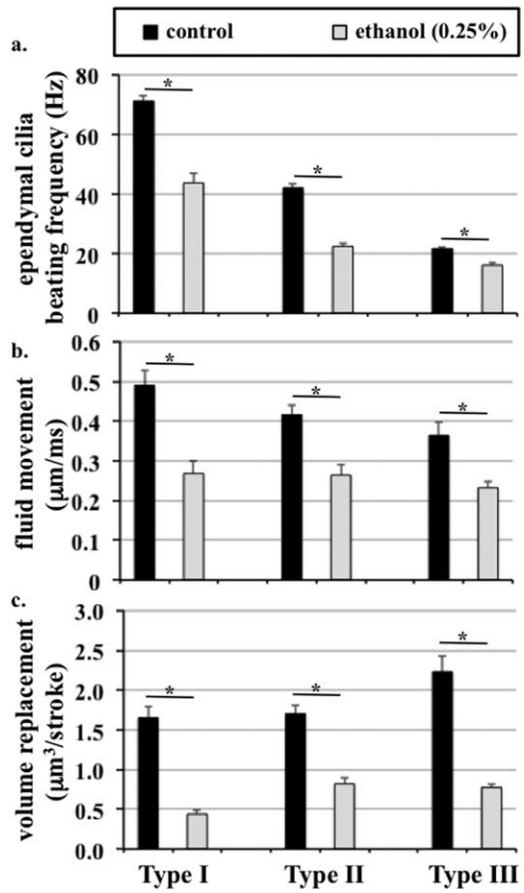


Fig. 5. Effects of alcohol on the dynamics of mouse third ventricle. The ex vivo brain slice was incubated without (control) or with (ethanol) alcohol for 5 min. **a:** Compared with control, alcohol treatment significantly decreased cilia beating frequency. **b:** This resulted in a decrease in fluid movement, as indicated by speed of fluid movement surrounding the ependymal cilia. **c:** Further calculation of the volume replacement and cilia beating indicated that, compared with control, alcohol significantly decreased volume replacement for each stroke of cilia beating. This indicates that alcohol not only decreases the frequency of ependymal cilia beating but also reduces the efficiency of each cilia stroke. At least 10 independent preparations were used for each ependymal cell type and treatment group.

ependymal cilia beating (Fig. 5a), resulting in a significant decrease in fluid movement velocity around ependymal cilia (Fig. 5b). Because of the transparency of the fluid media, we used nanobeads to guide us in measuring the speed of the fluid movement (Supp. Info. Movie 6). Given the fluid movement velocity, we estimated the volume replacement for each stroke of cilia beating efficiency. Our calculation indicated that ethanol not only repressed cilia beating frequency but also decreased the efficiency of ependymal cilia to move fluid per each stroke (Fig. 5c).

### Calcium Signaling by Ependymal Cilia Can Be Altered by Alcohol

Fluid-shear stress resulting from fluid movement above a layer of cells can generate intracellular calcium signaling (Abou Alaiwi et al., 2009b; Jin et al., 2013). We therefore examined the calcium signaling within the third ventricle (Fig. 6a). As expected, we did not see any apparent calcium oscillation in fixed brain sections (Supp. Info. Movie 7). In the absence (Supp. Info. Movie 8) or presence (Supp. Info. Movie 9) of ethanol, however, calcium oscillation was observed on ependymal cells. The frequency of calcium oscillation was not changed either by mock (PBS or control) or by ethanol treatment (Fig. 6b). Although the frequency of calcium signal in each ependymal cell type was unchanged, the amplitude of calcium signal was significantly repressed in the ethanol group compared with the control groups of each of the corresponding ependymal cell types (Fig. 6c).

## DISCUSSION

We report here for the very first time that there are three distinct types of ependymal cells uniquely and specifically positioned within the third ventricle. We classified them based on their cilia beating frequency as type I (>60Hz), type II (30–60 Hz), and type III (<30 Hz). The beat frequency for each type of ependymal cilia is age independent. We also report here that ependymal cells are characterized by calcium oscillations, the frequency and amplitude of which are the same in all ependymal cell types. Our chemical testing indicates that alcohol has a profound effect on the beating frequency of the ependymal cilia, resulting in a significant decrease in fluid movement and volume replacement. Although alcohol did not change the frequency of calcium oscillation in the ependymal cells, the amplitude of calcium oscillation was significantly repressed.

Even with the advancement in the technology of high-speed digital imaging (Lechtreck et al., 2009), there has been no report on different types of ependymal cells. We thus believe that our study is the first to identify distinct ependymal cilia, which is fundamentally important to gain basic understanding of ependymal physiology. For example, many substances that are known to alter cilia beating (Sisson et al., 1991; Sisson, 1995) may fail to show an effect in ependymal cells, especially when ependymal cells are randomly analyzed (Smith et al., 2013). To validate our point further, 1% ethanol was reported to have no effect on the beating frequency of ependymal cilia (Smith et al., 2013). After we classified the ependymal cilia into three types, however, our data clearly showed that ethanol as low as 0.25% had a definitive effect on ependymal cilia beating frequency.

Our study also revealed a unique aspect of calcium signaling in ependymal cells. It was previously thought that cardiac myocytes were the only cells that naturally have a pacing calcium oscillation. We used a florescence high-speed digital imaging system to demonstrate that, like myocytes, ependymal cells also have an oscillating intracellular calcium pattern. The frequency and amplitude of this calcium oscillation are similar among all three types of ependymal cells, indicating that the calcium may reflex functional states rather than types of the ependymal

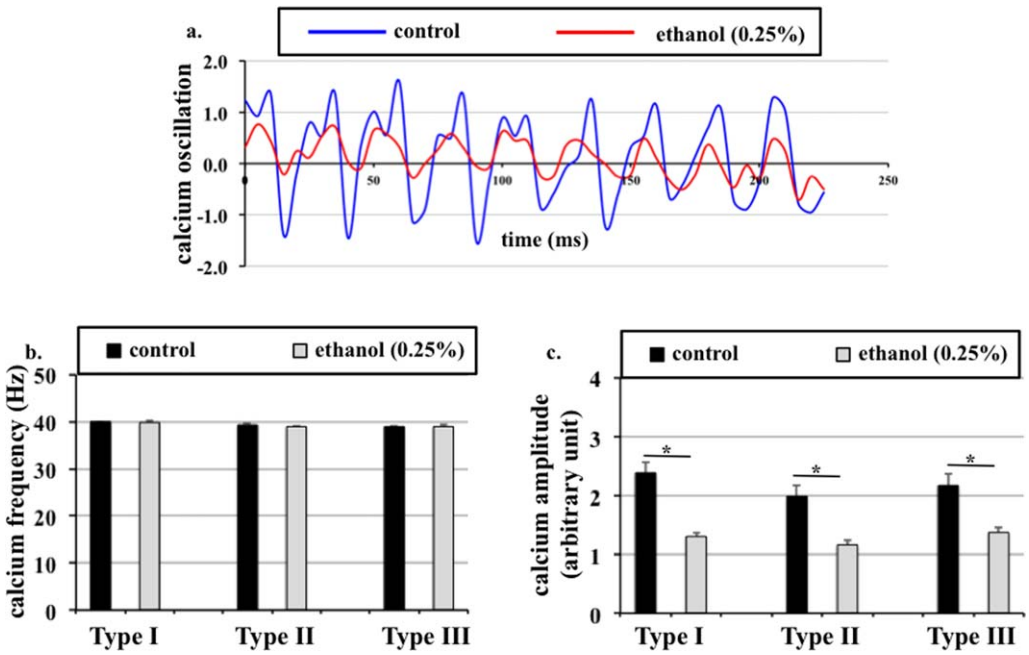


Fig. 6. Effects of alcohol on calcium oscillation in mouse brain ependymal cells. After being loaded with calcium indicator fluo-2, the ex vivo brain slice was incubated without (control) or with (ethanol) alcohol for 5 min. **a:** Intracellular calcium of ependymal cells was measured every 5 msec, as indicated by the representative blue and red lines. **b:** There was no difference in calcium oscillation frequency between control and alcohol-treated groups. **c:** However, the amplitude of calcium oscillation was significantly repressed in alcohol-treated groups compared with control groups in all types of ependymal cells. At least five independent preparations were used for each ependymal cell type and treatment group. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cells. Furthermore, the frequencies of ciliary beating and calcium oscillation on ependymal cells might not be associated with one another. Consistent with this idea, an ethanol-induced decrease in ciliary beating frequency did not alter the frequency of calcium oscillation.

Despite the fact that the characteristics of cilia beating are very different among these three types of ependymal cilia, it is important to note that our understanding of these cells is just starting to unfold. For example, cilostazol could differentially increase cilia beating frequency in type II but not in type I or type III cells (Supp. Info. Fig. 1). Cilostazol is a specific inhibitor for phosphodiesterase-3, an enzyme that metabolizes cAMP to AMP, and it also regulates intracellular calcium (Kawanabe et al., 2012). It has been known that cAMP and calcium could regulate cilia beating frequency (Nguyen et al., 2001; Monkkonen et al., 2008; Genzen et al., 2009), but the differential effect of cilostazol and many other pharmacological agents requires further in-depth study to advance our understanding of the molecular and cellular biology of different ependymal cell types.

It is also worth mentioning that, among the three types of ependymal cells, type III cells were the most efficient at moving fluid volume with each ciliary stroke. Although their frequency of ciliary beating was the slowest, type III cells had the largest angle of stroke. The angle of stroke might therefore contribute significantly to moving fluid volume. However, it is important to mention that ciliary beat frequency was also a critical contributing factor in moving fluid volume, as seen with the ethanol treatment. Treatment with ethanol decreased fluid replacement significantly, primarily because of a slowing of ciliary beating.

Alcohol can produce a variety of detrimental effects in the central nervous system, leading to a wide range of impairments. Within minutes of alcohol consumption, the alcohol in the CSF reaches the same level as that in the blood (Kiianmaa and Virtanen, 1978; Agapejev et al., 1992; Huang and Huang, 2007). However, the effect of alcohol on each type of ependymal cell had never before been examined, although abnormal ependymal cilia are associated with ventricle enlargement associated with hydrocephalus (Banizs et al., 2005; Baas et al., 2006; Wodarczyk et al., 2009; Tissir et al., 2010). Consistent with this notion, the brains of alcoholics are known to have an increase in the size of the ventricles, causing hydrocephalus ex vacuo (de la Monte, 1988). The use of 0.25% of ethanol in our study was within the range of alcohol levels in the CSF as observed in humans and various animal models (Kiianmaa and Virtanen, 1978; Agapejev et al., 1992; Huang and Huang, 2007). Thus, our study also reflects a serious clinical implication in alcohol abusive behavior with regard to ventricle-lining ependymal cells, in addition to providing fundamental basic

scientific understanding of ependymal cilia and calcium signaling.

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# Primary Cilium Regulates CaV1.2 Expression Through Wnt Signaling

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Primary cilia are sensory organelles that provide a feedback mechanism to restrict Wnt signaling in the absence of endogenous Wnt activators. Abnormal Wnt signaling has been shown to result in polycystic kidney disease (PKD) although the exact mechanism has been debated. Previously, we reported that the calcium channel CaV1.2 functions in primary cilia. In this study, we show that CaV1.2 expression level is regulated by Wnt signaling. This occurs through modulation of mitochondrial mass and activity resulting in increased reactive oxygen species which generate oxidative DNA lesions. We found that the subsequent cellular DNA damage response triggers increased CaV1.2 expression. In the absence of primary cilia where Wnt signaling is upregulated, we found that CaV1.2 is overexpressed as a compensatory mechanism. We show for the first time that CaV1.2 knockdown in zebrafish results in classic primary cilia defects including renal cyst formation, hydrocephalus, and left-right asymmetry defects. Our study shows that suppressed Wnt signaling prevents CaV1.2 expression ultimately resulting in PKD phenotypes. Thus, CaV1.2 expression is tightly regulated through Wnt signaling and plays an essential sensory role in primary cilia necessary for cellular homeostasis.

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Wnt signaling is an important regulator of cellular development and proliferation. In the absence of Wnt ligands, a complex consisting of Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3β (GSK3β) induces β-catenin for ubiquitylation by SCF E3 ligases and eventual proteasomal degradation (Aberle et al., 1997). Wnt signal transduction occurs when secreted Wnt ligands bind Frizzled receptors resulting in phosphorylation of LRP5/6. The Axin-APC-GSK3β complex is then recruited to LRP5/6 at the cell membrane which prevents β-catenin from being degraded. The accumulated β-catenin translocates to the nucleus and activates transcription of Wnt target genes (Muntean et al., 2012).

Primary cilia are non-motile sensory organelles present as a single copy on most differentiated cells in the body. Calcium signaling through primary cilium is essential for renal epithelial homeostasis (Nauli et al., 2003; Jin et al., 2013). Cilia extend from the cell surface through the basal body via intraflagellar transport (Moyer et al., 1994). The most common pathologies resulting from cilia dysfunction include polycystic kidney (Wilson, 2004), hypertension (Nauli et al., 2008; AbouAlaiwi et al., 2009), aneurysm (Aboualaiwi et al., 2013), hydrocephalus (Carter et al., 2012), and left-right asymmetry defects (Norris, 2012).

Abnormal Wnt signaling has also been linked to polycystic kidney disease (PKD) (Lancaster et al., 2009). For example, increased cytosolic and nuclear β-catenin accumulation has been shown in various cilia mutant cells (Gerdes et al., 2007; Lancaster et al., 2011). Thus, primary cilia are thought to provide a feedback mechanism that restricts Wnt signaling in the absence of appropriate ligands (Gerdes et al., 2007; Lancaster et al., 2009, 2011).

We recently showed that voltage-gated L-type calcium channel CaV1.2 localized to primary cilia in renal epithelia (Jin et al., 2013). Because Wnt signaling has also been reported to modulate mitochondrial physiology (Yoon et al., 2010), we hypothesized that primary cilia play a role in Wnt regulation of mitochondria through CaV1.2. We show that although CaV1.2 is not required for cilia formation, Wnt increases mitochondria mass and activity in CaV1.2 deficient renal epithelial cells. This increases mitochondria reactive oxidative species (ROS) and DNA damage, resulting in PKD phenotypes. Thus, our study suggests that primary cilia may play a role in CaV1.2 expression level through Wnt regulation of mitochondria.

## Materials and Methods

The experimental use of zebrafish was approved by The University of Toledo's Institutional Animal Care and Use Committee (IACUC). The use of lentiviral components was approved by the Institutional Biosafety Committee of The University of Toledo.

### Cell culture

Immortalized mouse renal epithelial wild-type and *Tg737*$^{orpk/orpk}$ cells were cultured in Dulbecco's Modified Eagle Medium (Corning Cellgro) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah) and 1% penicillin/streptomycin (Corning Cellgro) at 39°C in 5% $CO_2$, as previously described (Aboualaiwi et al., 2013). Prior to experiments, cells were treated with 100 ng/ml recombinant Wnt3a (R&D Systems, Minneapolis, MN) for 3 days and serum starved for 24 h.

### RNAi knockdown cells

shRNA lentiviral vectors specific to *Cacna1c* (*Origene*; pGFP-C-shLenti clone ID: TL500242) were transfected into HEK293T cells. Viral supernatants were collected after 48 h, centrifuged, and passed through a 0.45 μm filter. Cells were then spin-inoculated with pseudoviral particles containing 8 μg/ml polybrene at 2,500 rpm for 30 min at 30°C and then cultured for up to 1 week. CaV1.2 knockdown was verified through Western blot analysis.

Brian S. Muntean and Xingjian Jin contributed equally to this work.

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TABLE 1. shRNA sequences

| Descriptions | Sequences |
|---|---|
| Scrambled control | 5′-TGACCACCCTGACCTACGGCGTGCAGTGC-3′ |
| *Cacna1c* | 5′-TCAGAAGTGCCTCACTGTTCTCGTGACCT-3′ |

Stable knockdown cell lines were obtained through puromycin selection. The following shRNA sequences were used (Table 1).

### Immunostaining studies

Cells were grown to confluence on collagen-coated glass coverslips and differentiated in serum-free media for 24 h. Cells were then fixed in 4% paraformaldehyde in PBS containing 2% sucrose, permeabilized in 10% triton X-100, incubated sequentially with primary followed by secondary antibodies for 1 h each, and finally mounted on a slide with DAPI hard set mounting media (Vector Laboratories, Burlingame, CA). The following primary antibody dilutions were used: acetylated-α-tubulin 1:10,000 (Sigma-Aldrich, St. Louis, MO) and CaV1.2 1:50 (Alomone Labs, Jerusalem, Israel). Anti-mouse Texas Red and anti-rabbit FITC fluorescent conjugated secondary antibodies were used at 1:500 (VectorLabs).

### Mitochondrial studies

MitoTracker Green FM and MitoTracker Red CMXRos (Cell Signaling Technology) were incubated with cells at 100 nM for 30 min at 37°C. MitoSOX (Life Technologies) was incubated with cells at 5 μM for 10 min at 37°C. After staining, cells were washed three times with PBS and analyzed immediately through microscopy or flow cytometry. For microscopic analysis, cells were grown on custom glass-bottom cell culture plates and imaged under a Nikon Eclipse TE2000-U microscope controlled by MetaMorph software with a 100× objective lens. For flow cytometry studies, cells were detached with trypsin, washed, and analyzed.

### DNA damage assessment

Oxidative DNA lesions were detected with an 8-oxoguanine antibody (Santa Cruz). Detached cells were fixed in 4% formaldehyde for 10 min at 37°C and permeabilized in ice-cold 90% methanol for 30 min on ice. After washing with PBS, cells were incubated in PBS containing anti-8-oxoguanine antibody (1:50), 0.5% Tween-20, and 5% FBS for 1 h. Cells were washed and incubated in PBS containing anti-mouse Texas Red antibody (1:500), 0.5% Tween-20, and 5% FBS for 1 h. Cells were then washed and analyzed with flow cytometer.

### Mitochondrial DNA and mRNA measurement

Total cellular DNA was obtained using the DNeasy Blood & Tissue Kit (Qiagen) and used for detection with PCR primers listed below to quantify the nuclear (*18S rRNA*) to mitochondrial DNA (*Coi*) ratio as described (Brown and Clayton, 2002; Bai et al., 2004). Total cellular RNA was obtained using TRIzol (Life Technologies) and reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR detection of expression genes was performed using the primers listed below comparing mitochondrial encoded oxidative phosphorylation genes (*ATP5γ1* and *CytC*) to nuclear encoded *β-Actin* as described (Yoon et al., 2010) (Table 2).

### Western blot analysis

Cells were scraped from culture plates in the presence of RIPA buffer supplemented with Complete Protease Inhibitor (Roche,

TABLE 2. Primer sequences

| Descriptions | Sequences |
|---|---|
| *Coi* F | 5′-GCCCCAGATATAGCATTCCC-3′ |
| *Coi* R | 5′-GTTCATCCTGTTCCTGCTCC-3′ |
| *18S rRNA* F | 5′-TAGAGGGACAAGTGGCGTTC-3′ |
| *18S rRNA* R | 5′-CGCTGAGCCAGTCAGTGT-3′ |
| ATP5γ1 F | 5′-AGTTGGTGTGGCTGGATCA-3′ |
| ATPγ1 R | 5′-GCTGCTTGAGAGATGGGTTC-3′ |
| CytC F | 5′-GGAGGCAAGCATAAGACTGG-3′ |
| CytC R | 5′-TCCATCAGGGTATCCTCTCC-3′ |
| *β-actin* F | 5′-TGTTACCAACTGGGACGACA-3′ |
| *β-actin* R | 5′-GGGGTGTTGAAGGTCTCAAA-3’ |

New York, NY), incubated on ice with frequent vortexing, and centrifuged. Supernatants were subjected to protein quantification and PAGE on 6–10% SDS gels followed by wet transfer to PVDF membranes and detection using β-catenin 1:1,000, CaV1.2 1:200, NF-κB p65 1:200, and GAPDH 1:1,000 (Cell Signaling Technology, Danvers, MA).

### Zebrafish

Adult wild-type AB zebrafish were obtained from the Zebrafish International Resource Center (Eugene, OR) and used for breeding. Embryos were injected with 1 mM antisense translation blocking morpholino oligos (MO; GeneTools) at the 1–2 cell stage. Zebrafish embryos were then cultured at 28.5°C in sterile egg water (Muntean et al., 2010). The following MO sequences were used: *control MO:* 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′, *cav1.2 MO:* 5′-ACA TGT TTT TGC TTT CAT TTA CCA T-3′, *pkd2 MO:* 5′-AGG ACG AAC GCG ACT GGA GCT CAT C-3′. Knockdown of CaV1.2 was verified through Western blot analysis. Briefly, zebrafish embryos were dechorionated at 28 h postfertilization and homogenized in RIPA buffer to obtain protein extracts. Western was performed on 50 μg total protein using CaV1.2 (1:200) and GAPDH (1:1,000) antibodies.

Histological examination was used to measure renal cyst formation and hydrocephalus at 3 days postfertilization. Embryos were fixed in a PBS solution containing 4% paraformaldehyde and 2% sucrose overnight at 4°C, dehydrated through an ethanol gradient, and embedded in JB4 resin (Polysciences, Inc., Warrington, PA) as specified in manufacturer's protocol. A Reichert Jung microtome was used to cut 5 μm sections which were subsequently hematoxylin and eosin stained. Heart looping was assessed at 48 h postfertilization by positioning zebrafish on their dorsal axis and recording heart beat to reveal the respective relative locations of the atrium and the ventricle.

### Data analysis

Data are reported as the mean ± standard error of the mean. All image analysis was performed using ImageJ. All flow cytometry data were analyzed with BD Accuri C6 software and were presented without any compensation gating. All data were analyzed using IBM SPSS Statistics Version 21 software by performing the student *t*-test for two group comparison or ANOVA test followed by Tukey's post-test for three or more group comparison. Statistical significance is reported with a statistical power greater than 0.8 at $P < 0.05$.

## Results

### CaV1.2 is not required for primary cilia assembly

We recently reported that the voltage gated L-type calcium channel CaV1.2 localized to primary cilia in bovine LLCPK cells (Jin et al., 2013). We performed immunostaining to verify this finding in mouse renal epithelial cells (Fig. 1). The mouse *Tg737*$^{orpk/orpk}$ cell line contains a hypomorphic mutation in an intraflagellar transport gene (*Ift88*) that is required for cells

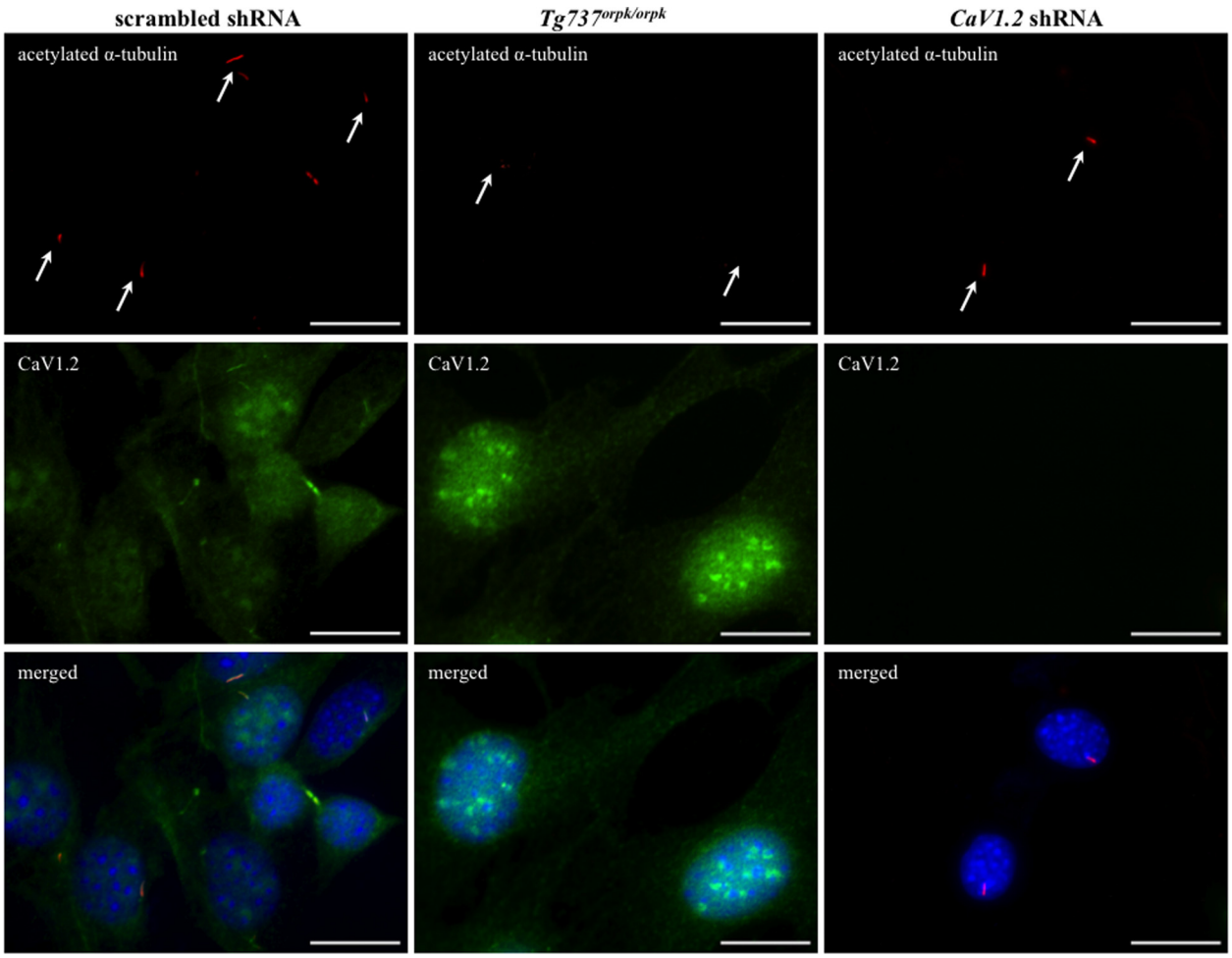


**Fig. 1. Localization of CaV1.2 to renal epithelial cilia is not required for primary cilia assembly. Immunofluorescence revealed that CaV1.2 localized to primary cilia in renal epithelial cells (scrambled shRNA) when compared with cilia-deficient cells (Tg737$^{orpk/orpk}$). The presence of primary cilia was confirmed in CaV1.2 shRNA cells. Acetylated-α-tubulin was used as a ciliary marker. Arrow indicates the presence of primary cilium, except in cilia-deficient cells. Bar = 20 μm.**

to assemble primary cilia (Moyer et al., 1994). Thus, the *Tg737*$^{orpk/orpk}$ system is a well-established model for studying cells without longer primary cilia, as verified through our immunostaining. We next asked if CaV1.2 played a role in primary cilia assembly. We generated a stable CaV1.2 shRNA knockdown mouse renal epithelial cell line and immunostaining studies revealed that primary cilia were similar to that of scrambled shRNA.

### Wnt3a induces mitochondrial biogenesis in CaV1.2-deficient but not cilia-deficient cells

Wnt signaling has recently been reported to regulate mitochondrial physiology (Yoon et al., 2010). To assess mitochondrial mass, cells were stained with Mito Tracker Green (MTG) and observed live using fluorescence microscopy. When treated with recombinant Wnt3a, mitochondrial mass increased (Fig. 2a). However, the mitochondrial mass in *Tg737*$^{orpk/orpk}$ cells was unchanged after Wnt3a treatment. We next performed this experiment in CaV1.2 shRNA cells and the results were similar to that of the scrambled control. To quantify these findings, MTG fluorescence was recorded using flow cytometry which confirmed our fluorescent observation (Fig. 2b). Our MTG studies were further validated using a common technique by comparing mitochondrial DNA (*Coi*) to nuclear DNA (*18S rRNA*) (Brown and Clayton, 2002; Bai et al., 2004). As expected, Wnt3a did indeed statistically increase mitochondrial biogenesis in scrambled and CaV1.2 shRNA but not in *Tg737*$^{orpk/orpk}$ cells (Fig. 2c). Our immunofluorescence study showed that Wnt3a did not alter CaV1.2 localization to cilia (Table 3).

### Wnt3a increases mitochondrial activity in CaV1.2-deficient cells while decreasing activity in cilia-deficient cells

We next asked if Wnt3a would have an effect on mitochondrial oxidative phosphorylation (activity) in *Tg737*$^{orpk/orpk}$ cells. Similar to before, we stained cells with Mito Tracker Red (MTR). Unlike MTG, MTR staining is dependent on the mitochondrial membrane potential (Poot and Pierce, 2001; Pendergrass et al., 2004). Therefore, increased staining

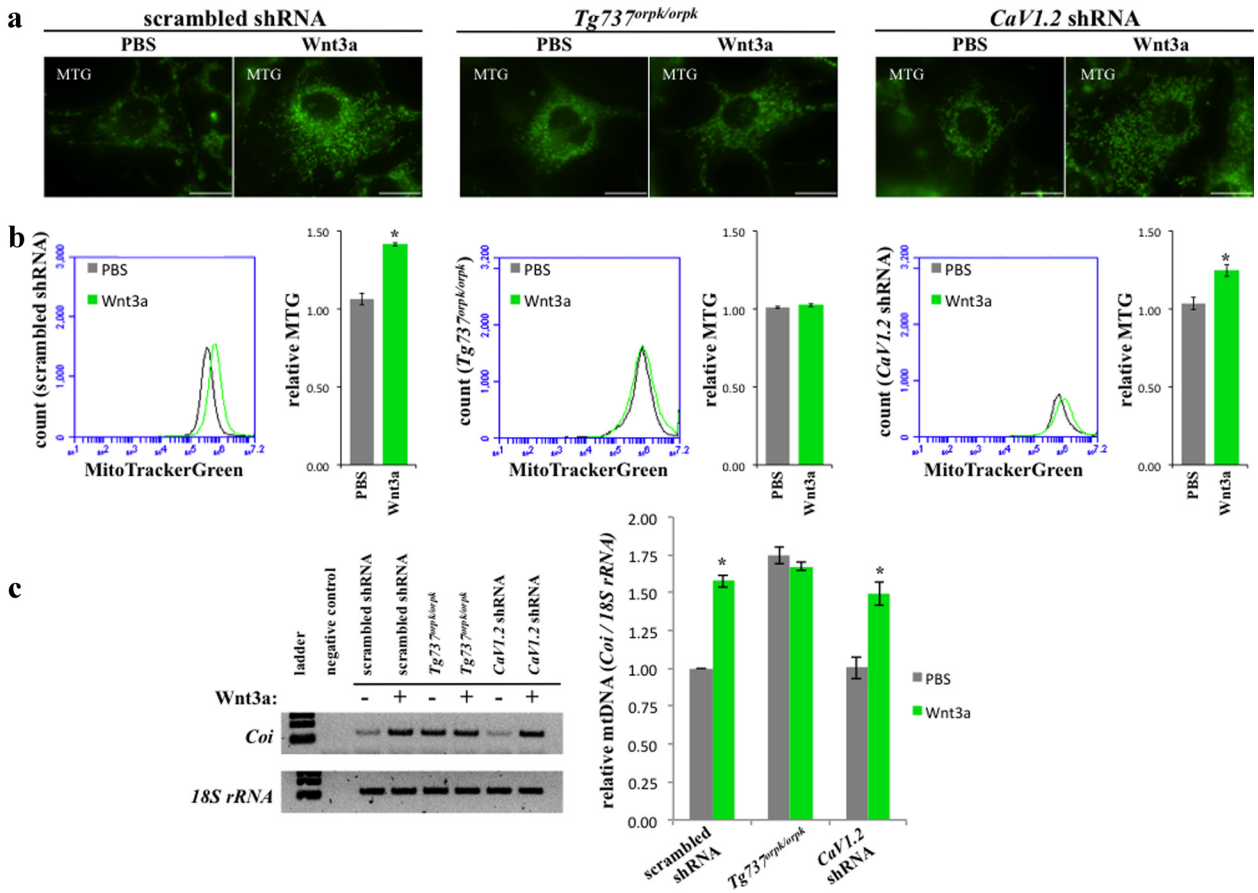


**Fig. 2. Wnt3a induces mitochondrial biogenesis in CaV1.2 shRNA but not Tg737$^{orpk/orpk}$ cells. a: Mitochondrial mass was assessed by staining cells with Mito Tracker Green. Wnt3a was found to induce mitochondrial mass in scrambled and CaV1.2 shRNA cells but had no effect on Tg737$^{orpk/orpk}$ when examined using fluorescence microscopy (bar = 20 μm). b: Results were quantified using flow cytometry. c: Mitochondrial DNA was measured using PCR by taking the ratio of a mitochondrial gene (Coi) to a nuclear gene (18S rRNA) (N = 3).**

correlates to increased oxidative phosphorylation. As expected, Wnt3a increased MTR staining in scrambled and CaV1.2 shRNA cells when observed using fluorescence microscopy (Fig. 3a). However, mitochondrial activity decreased in *Tg737*$^{orpk/orpk}$ cells. We again quantified our findings using flow cytometry (Fig. 3b). Wnt3a significantly increased mitochondrial activity in scrambled and CaV1.2 shRNA while significantly decreasing activity in *Tg737*$^{orpk/orpk}$ cells. To verify these results, we compared expression of two key mitochondrial encoded oxidative phosphorylation genes (ATP Synthase 5γ1 and Cytochrome *c*) relative to that of nuclear encoded β-actin (Fig. 3c).

TABLE 3. CaV1.2 ciliary localization

| | % CaV1.2 localization to cilia | N |
|---|---|---|
| PBS (control) | | |
| Scramble shRNA | 91.1 | 45 |
| *Tg737*$^{orpk/orpk}$ | 91.7 | 36 |
| *CaV1.2* shRNA | 0.0 | 41 |
| Wnt3a (100 ng/ml) | | |
| Scramble shRNA | 90.4 | 52 |
| *Tg737*$^{orpk/orpk}$ | 92.3 | 39 |
| *CaV1.2* shRNA | 0.0 | 46 |

### Wnt3a increases ROS and DNA damage in CaV1.2-deficient but not in cilia-deficient cells

An inevitable consequence of oxidative phosphorylation is the generation of reactive oxygen species (ROS) (Boveris et al., 1972; Boveris and Chance, 1973). MitoSOX is a cell permeable red fluorescent indicator specific for mitochondrial ROS. We therefore stained cells with MitoSOX and observed a significant increase in mitochondrial ROS in scrambled and CaV1.2 shRNA after treatment with Wnt3a (Fig. 4a). A significant decrease in staining was observed in *Tg737*$^{orpk/orpk}$ cells (Fig. 4b). Genomic DNA can be damaged by ROS to form DNA lesions resulting from mismatched repairs (Kasai et al., 1984). Thus, we quantified the levels of 8-Oxoguanine, a common DNA lesion formed by mismatched Adenine (Kasai, 1997). Treatment with Wnt3a was found to increase 8-Oxoguanine in scrambled and CaV1.2 shRNA while no change was observed in *Tg737*$^{orpk/orpk}$ cells (Fig. 4c).

### Cilia modulates Wnt signaling to regulate CaV1.2 expression

As previously reported, Wnt3a treatment induced β-catenin expression in all cells (Aberle et al., 1997). We confirmed this in our system, including in *Tg737*$^{orpk/orpk}$ and CaV1.2 shRNA cells (Fig. 5). Consistent with previous study (Corbit et al., 2008),

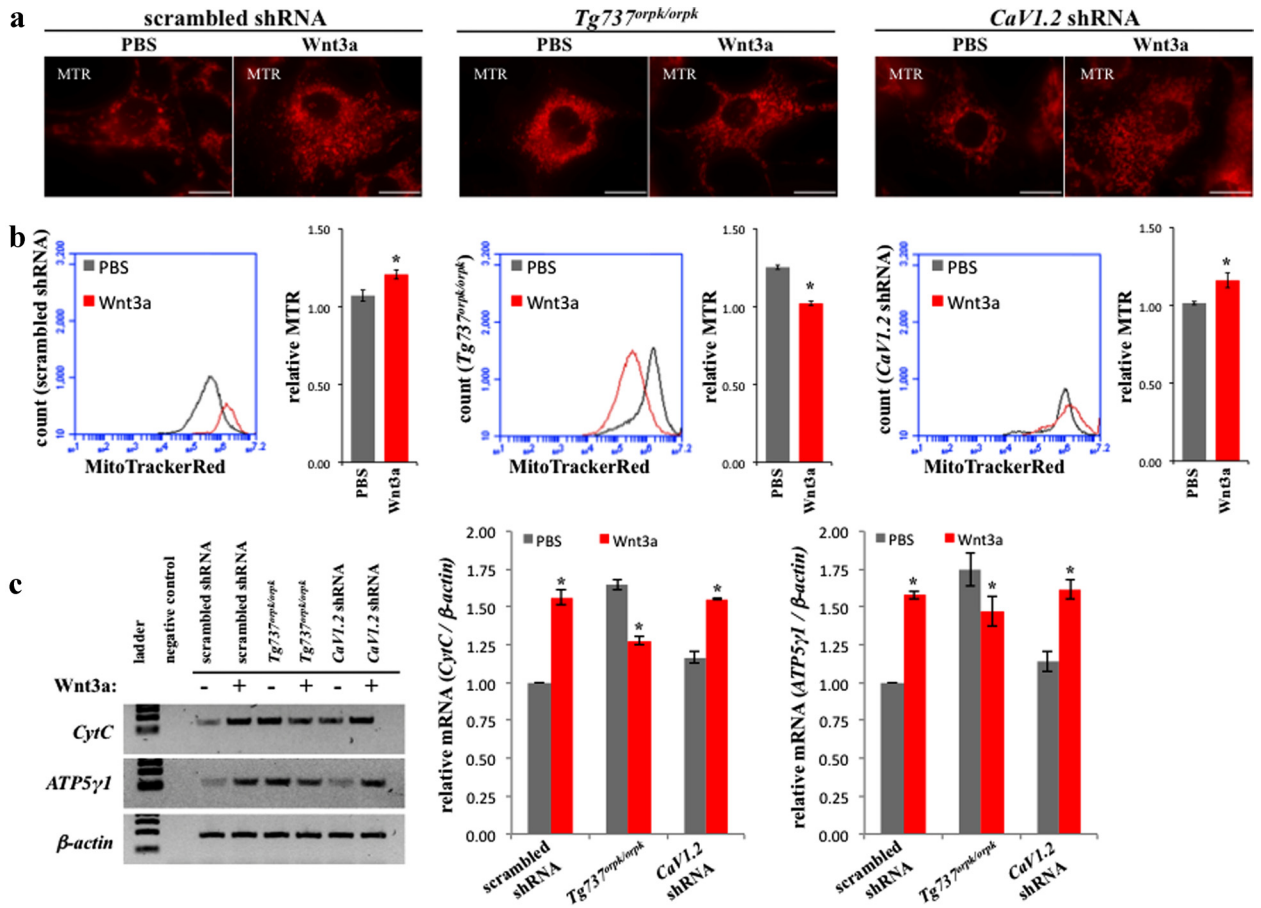


**Fig. 3. Wnt3a increases mitochondrial activity in CaV1.2 shRNA but decreasing activity in Tg737$^{orpk/orpk}$ cells. a: Mitochondrial oxidative phosphorylation was used to indicate activity through Mito Tracker Red staining. Wnt3a was found to increase oxidative phosphorylation in scrambled and CaV1.2 shRNA cells while decreasing oxidative phosphorylation in Tg737$^{orpk/orpk}$ when examined using fluorescence microscopy (bar = 20 μm). b: Results were quantified using flow cytometry. c: Two mitochondrial mRNAs encoded oxidative phosphorylation genes (CytC and ATP5γ1) were measured using PCR normalized to nuclear encoded β-actin (N = 3).**

*Tg737$^{orpk/orpk}$* cells showed a higher basal level of β-catenin than control. Further, Wnt3a treatment increased CaV1.2 expression in scrambled shRNA while decreasing CaV1.2 in *Tg737$^{orpk/orpk}$* cells. Of note is that CaV1.2 expression was not detectable in CaV1.2 shRNA cells, confirming knockdown of CaV1.2 in our stable cell line.

The DNA damage response (DDR) is a cellular mechanism to recover from DNA lesions, such as 8-Oxoguanine (Kasai et al., 1984; Jackson and Bartek, 2009). One arm of this cell survival pathway is the activation of nuclear factor κB p65 (NFκB p65) (Janssens and Tschopp, 2006). Through Western blot analysis, we also found that Wnt3a induced NFκB p65 expression in scrambled and CaV1.2 shRNA (Fig. 5). On the other hand, *Tg737$^{orpk/orpk}$* cells expressed a high basal level of NFκB p65 which decreased in response to Wnt3a. In addition, CaV1.2 expression was found to correlate with NFκB p65.

### CaV1.2 knockdown zebrafish develop PKD phenotypes

We have shown that CaV1.2 localizes to primary cilia and have now elucidated the mechanism by which CaV1.2 expression is regulated in renal epithelial cells. To assess the biological significance of CaV1.2 expression, we used antisense morpholinos to knockdown CaV1.2 in zebrafish. Knockdown of the ciliary calcium channel polycystin-2 in zebrafish has been reported to result in PKD phenotypes including renal cyst formation, hydrocephalus, and left-right asymmetry (Obara et al., 2006). Our study showed that knockdown of *pkd2* increased CaV1.2 expression (Fig. 6). This slight increase in CaV1.2 was significant compared to the control morpholino. Interestingly, similar phenotypes were observed in CaV1.2 morpholino (*cav1.2 MO*) zebrafish. Compared with a non-specific control morpholino (*control MO*) injection, *cav1.2 MO* zebrafish developed renal cysts (Fig. 7a), hydrocephalus (Fig. 7b) and various heart-looping defects (Fig. 7c). As generally accepted (Bakkers, 2011), left-right asymmetry was assessed by measuring the relative position of the cardiac atrium and ventricle with respect to the dorsal axis (Supplemental Movie 1).

## Discussion

Non-motile primary cilia have been found to play a critical role in Wnt signaling by restricting β-catenin accumulation. Overexpression of polycystin-1 (a ciliary signaling receptor) inhibits GSK3β and stabilizes β- catenin (Kim et al., 1999).

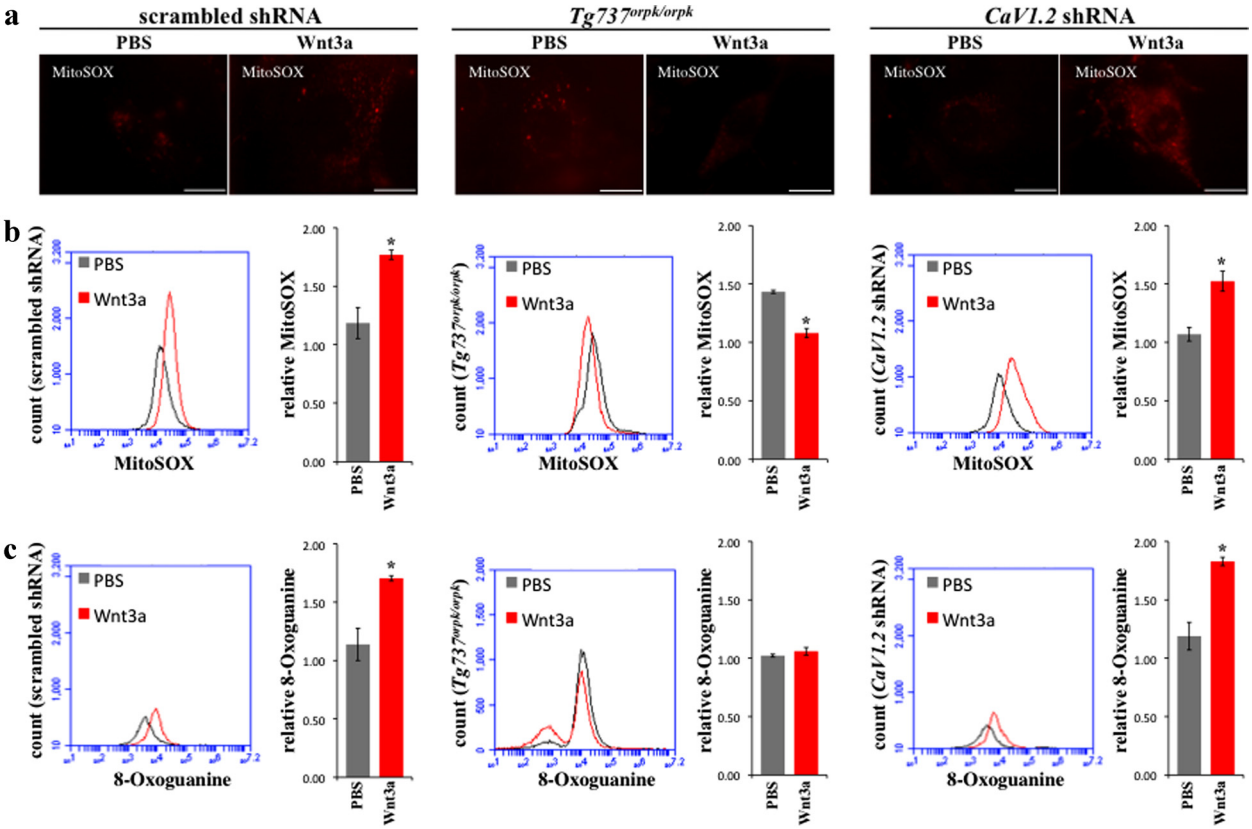


**Fig. 4. Wnt3a increases ROS and DNA damage in CaV1.2 shRNA but not in Tg737$^{orpk/orpk}$ cells. a: Mitochondrial ROS was assessed by staining cells with MitoSOX. Wnt3a was found to increase ROS in scrambled and CaV1.2 shRNA cells while decreasing ROS in Tg737$^{orpk/orpk}$ when examined using fluorescence microscopy (bar = 20 μm). b: Results were quantified using flow cytometry. c: Wnt3a increased formation of the oxidative DNA lesion 8-Oxoguanine in scrambled and CaV1.2 shRNA cells but had no effect on Tg737$^{orpk/orpk}$ cells (N = 3).**

Polycystin-2 (encoded by *Pkd2*) is calcium channel forming protein found in primary cilia. In *Pkd2*$^{-/-}$ embryos, cilia length was found to be decreased while β-catenin was upregulated (Kim et al., 2009). Interestingly, transgenic mice overexpressing β-catenin also developed cystic kidneys (Saadi-Kheddouci et al., 2001). Further, LRP6$^{-/-}$ (a component of the Wnt receptor complex) mouse embryos die in utero with cystic kidneys (Pinson et al., 2000). Thus, primary cilia and Wnt signaling play a crucial role in PKD (Corbit et al., 2008).

Given that Wnt signaling also modulates mitochondrial physiology (Yoon et al., 2010), we examined the role of cilia in regards to mitochondria. *Tg737*$^{orpk/orpk}$ contains an intron insertion at the 3′ end of the intraflagellar transport 88 (*Ift88*) gene which results in a hypomorphic mutation that prevents ciliogenesis (Moyer et al., 1994). We used *Tg737*$^{orpk/orpk}$ cells as a model for a cilia-deficient system. Through immunostaining, we confirmed the absence of cilia in *Tg737*$^{orpk/orpk}$ cells compared with control (Fig. 1). The voltage-gated L-type calcium channel CaV1.2 also localized to primary cilia. We generated a stable CaV1.2 shRNA cell line and observed no changes in primary cilia compared with control. Further, treatment with Wnt3a had no effect on cilia number or length in scrambled or CaV1.2 shRNA cells (data not shown). Thus, CaV1.2 does not seem to play a role in ciliogenesis.

Mitochondrial biogenesis, oxidative phosphorylation, and generation of reactive oxidative species (ROS) were increased in response to Wnt3a in control renal epithelial cells (Figures 2–4). The elevated levels of oxidative stress increased the formation of DNA lesions and the cellular DNA damage response (DDR). An interesting aspect of this response was an increase in expression of CaV1.2 (Fig. 5). In CaV1.2 knockdown cells, Wnt3a induced a similar effect on mitochondria and DDR. This data suggests that CaV1.2 is a downstream effector in regard to Wnt signaling. In cilia-deficient cells, Wnt3a was unable to induce mitochondrial biogenesis and decreased mitochondrial activity, ROS production, and DDR. CaV1.2 was found to be overexpressed in cilia-deficient cells as a compensatory mechanism; however, its expression decreased following Wnt3a treatment. Therefore, cilia length plays a role in regulating CaV1.2 expression through modulation of Wnt signaling.

Defective primary cilia, indicated by either depletion of key ciliary proteins or fundamental changes in structure/length, results in PKD phenotypes (Wilson, 2004). Here we show that CaV1.2 is a biologically significant ciliary protein. In the absence of CaV1.2 in zebrafish (Fig. 6), PKD phenotypes including renal cyst formation, hydrocephalus, and left-right asymmetry defects were observed (Fig. 7). Moreover, CaV1.2 was found to be overexpressed in *pkd2* knockdown zebrafish. This is intriguing given that both CaV1.2 and PC2 are calcium channel forming proteins in the primary cilium, which further suggests a role for CaV1.2 in PKD pathogenesis. Therefore, CaV1.2 not only localizes to renal epithelial primary cilia, but it is also required for cilia function.

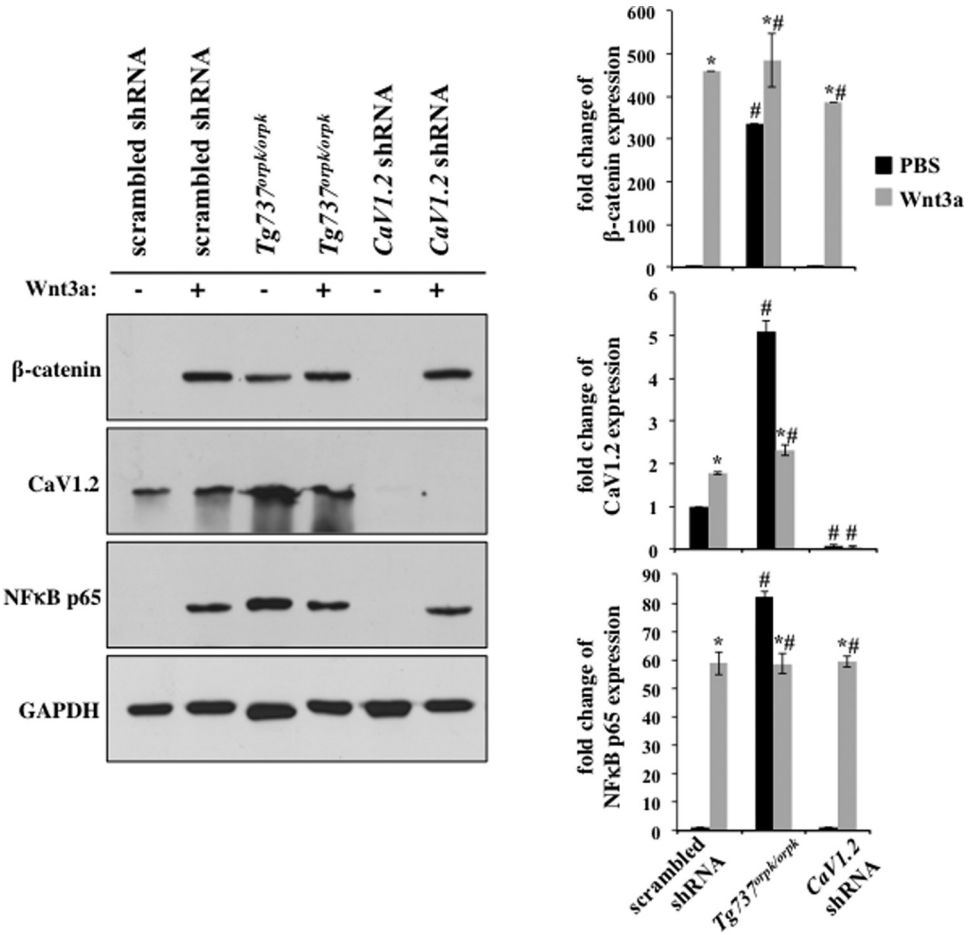


**Fig. 5. Cilia modulates Wnt signaling to regulate CaV1.2 expression. Western blotting was performed on cellular protein extracts. Wnt3a treatment increased β-catenin accumulation in all cells, however, Tg737$^{orpk/orpk}$ cells displayed high basal levels of β-catenin. NF-κB p65 was blotted as a readout for DNA damage response (DDR) to ROS induced DNA lesions. Wnt3a induced NF-κB p65 expression in scrambled and CaV1.2 shRNA cells but decreased NF-κB p65 in Tg737$^{orpk/orpk}$ cells. CaV1.2 was overexpressed in Tg737$^{orpk/orpk}$ cells. Wnt3a treatment induced CaV1.2 expression in scrambled shRNA while decreasing expression in Tg737$^{orpk/orpk}$ cells. Data normalized to GAPDH for analysis (N = 3). Asterisks indicate significant difference from the corresponding control group ($P < 0.05$). # signs denote significant difference from the corresponding scrambled shRNA group ($P < 0.05$).**

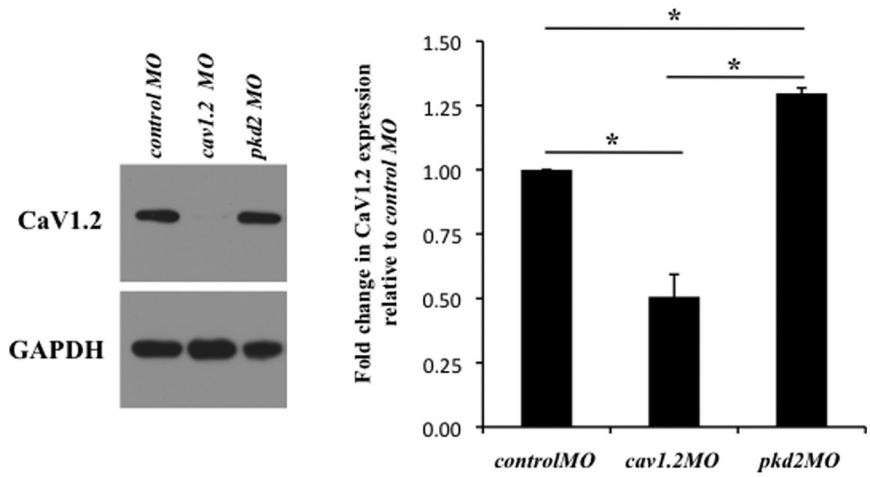


**Fig. 6. pkd2 knockdown increases CaV1.2 expression in zebrafish. Embryonic zebrafish protein extracts were obtained at 28 h postfertilization. Western blotting showed that cav1.2MO effectively reduced CaV1.2 expression compared with controlMO. pkd2MO zebrafish were used to further verify morpholino specificity. Results were quantified through one-way ANOVA with Tukey post-test. Statistical significance is reported with a mean difference at the 0.05 level and denoted with an asterisk (N = 3).**

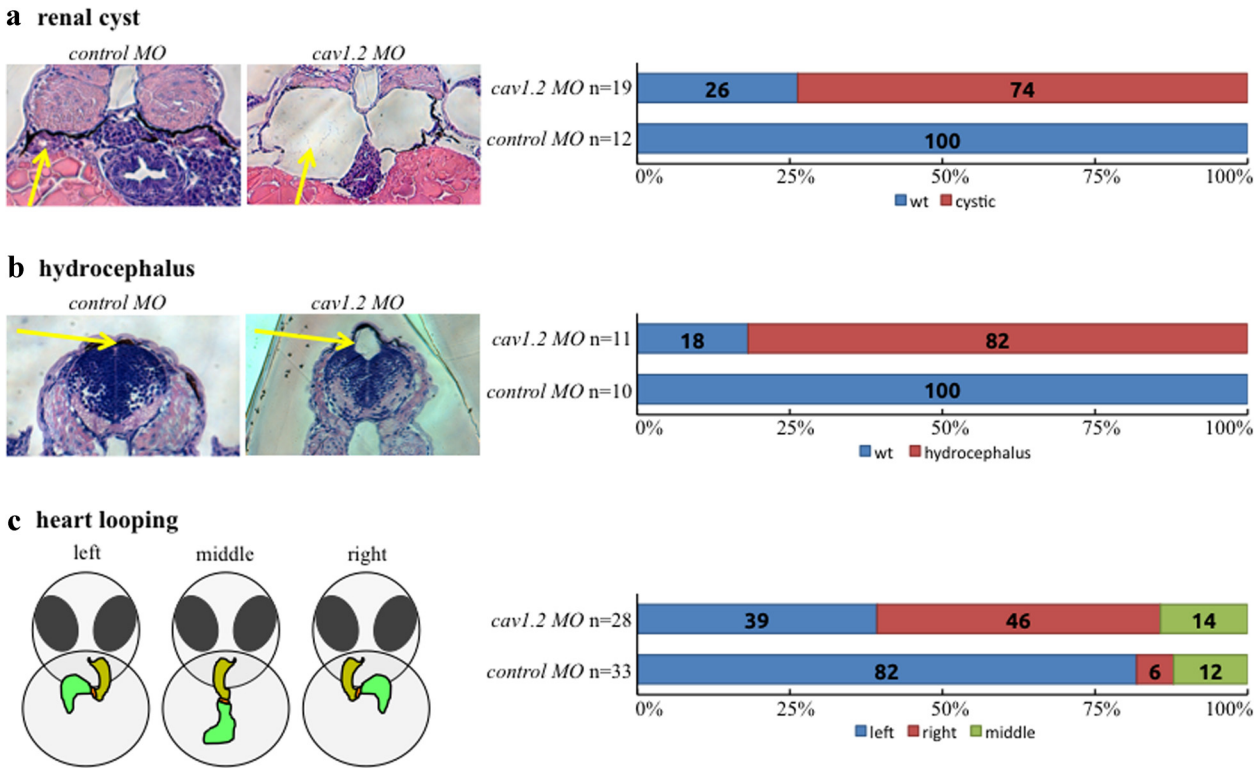


**Fig. 7. CaV1.2 knockdown zebrafish develop renal cysts, hydrocephalus, and left-right asymmetry defects. CaV1.2 was knocked down in zebrafish (cav1.2 MO) using morpholino microinjection and compared to a scrambled control morpholino (control MO) to assess phenotypes. Renal cyst formation (a) and hydrocephalus (b) were measured at 3 days postfertilization through standard H&E staining as indicated by arrows. Left-right asymmetry was determined by measuring heart looping (c) at 2 days postfertilization under live microscopy.**

Although it has been known that abnormal Wnt signaling leads to renal cyst formation (Lancaster et al., 2009), the underlying mechanism has been unclear. One proposed explanation is that Wnt signaling regulates renal cell proliferation and planar cell polarity to maintain renal tubule homeostasis (Happe et al., 2009). Our present study further suggests that cilia regulate Wnt signaling which ultimately controls CaV1.2 expression. Therefore, in the absence of CaV1.2, ciliary function is compromised leading to formation of renal cysts. In addition, our study also shows a previously unrecognized relationship between primary cilia and mitochondrial function. Overall, we propose that Wnt3a induces β-catenin accumulation in a cilia-dependent manner. This in turn increases mitochondrial biogenesis, oxidative phosphorylation, and generation of ROS that cause genomic DNA damage. The DDR triggers NFκB p65 expression ultimately resulting in increased CaV1.2 expression.

## Acknowledgments

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## Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Journal of Cellular Physiology



# Primary Cilium Regulates CaV1.2 Expression Through Wnt Signaling

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Primary cilia are sensory organelles that provide a feedback mechanism to restrict Wnt signaling in the absence of endogenous Wnt activators. Abnormal Wnt signaling has been shown to result in polycystic kidney disease (PKD) although the exact mechanism has been debated. Previously, we reported that the calcium channel CaV1.2 functions in primary cilia. In this study, we show that CaV1.2 expression level is regulated by Wnt signaling. This occurs through modulation of mitochondrial mass and activity resulting in increased reactive oxygen species which generate oxidative DNA lesions. We found that the subsequent cellular DNA damage response triggers increased CaV1.2 expression. In the absence of primary cilia where Wnt signaling is upregulated, we found that CaV1.2 is overexpressed as a compensatory mechanism. We show for the first time that CaV1.2 knockdown in zebrafish results in classic primary cilia defects including renal cyst formation, hydrocephalus, and left-right asymmetry defects. Our study shows that suppressed Wnt signaling prevents CaV1.2 expression ultimately resulting in PKD phenotypes. Thus, CaV1.2 expression is tightly regulated through Wnt signaling and plays an essential sensory role in primary cilia necessary for cellular homeostasis.

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Wnt signaling is an important regulator of cellular development and proliferation. In the absence of Wnt ligands, a complex consisting of Axin, adenomatous polyposis coli (APC), and glycogen synthase kinase 3β (GSK3β) induces β-catenin for ubiquitylation by SCF E3 ligases and eventual proteasomal degradation (Aberle et al., 1997). Wnt signal transduction occurs when secreted Wnt ligands bind Frizzled receptors resulting in phosphorylation of LRP5/6. The Axin-APC-GSK3β complex is then recruited to LRP5/6 at the cell membrane which prevents β-catenin from being degraded. The accumulated β-catenin translocates to the nucleus and activates transcription of Wnt target genes (Muntean et al., 2012).

Primary cilia are non-motile sensory organelles present as a single copy on most differentiated cells in the body. Calcium signaling through primary cilium is essential for renal epithelial homeostasis (Nauli et al., 2003; Jin et al., 2013). Cilia extend from the cell surface through the basal body via intraflagellar transport (Moyer et al., 1994). The most common pathologies resulting from cilia dysfunction include polycystic kidney (Wilson, 2004), hypertension (Nauli et al., 2008; AbouAlaiwi et al., 2009), aneurysm (Aboualaiwi et al., 2013), hydrocephalus (Carter et al., 2012), and left-right asymmetry defects (Norris, 2012).

Abnormal Wnt signaling has also been linked to polycystic kidney disease (PKD) (Lancaster et al., 2009). For example, increased cytosolic and nuclear β-catenin accumulation has been shown in various cilia mutant cells (Gerdes et al., 2007; Lancaster et al., 2011). Thus, primary cilia are thought to provide a feedback mechanism that restricts Wnt signaling in the absence of appropriate ligands (Gerdes et al., 2007; Lancaster et al., 2009, 2011).

We recently showed that voltage-gated L-type calcium channel CaV1.2 localized to primary cilia in renal epithelia (Jin et al., 2013). Because Wnt signaling has also been reported to modulate mitochondrial physiology (Yoon et al., 2010), we hypothesized that primary cilia play a role in Wnt regulation of mitochondria through CaV1.2. We show that although CaV1.2 is not required for cilia formation, Wnt increases mitochondria mass and activity in CaV1.2 deficient renal epithelial cells. This increases mitochondria reactive oxidative species (ROS) and DNA damage, resulting in PKD phenotypes. Thus, our study suggests that primary cilia may play a role in CaV1.2 expression level through Wnt regulation of mitochondria.

## Materials and Methods

The experimental use of zebrafish was approved by The University of Toledo's Institutional Animal Care and Use Committee (IACUC). The use of lentiviral components was approved by the Institutional Biosafety Committee of The University of Toledo.

### Cell culture

Immortalized mouse renal epithelial wild-type and *Tg737*$^{orpk/orpk}$ cells were cultured in Dulbecco's Modified Eagle Medium (Corning Cellgro) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, Utah) and 1% penicillin/streptomycin (Corning Cellgro) at 39°C in 5% $CO_2$, as previously described (Aboualaiwi et al., 2013). Prior to experiments, cells were treated with 100 ng/ml recombinant Wnt3a (R&D Systems, Minneapolis, MN) for 3 days and serum starved for 24 h.

### RNAi knockdown cells

shRNA lentiviral vectors specific to *Cacna1c* (*Origene*; pGFP-C-shLenti clone ID: TL500242) were transfected into HEK293T cells. Viral supernatants were collected after 48 h, centrifuged, and passed through a 0.45 μm filter. Cells were then spin-inoculated with pseudoviral particles containing 8 μg/ml polybrene at 2,500 rpm for 30 min at 30°C and then cultured for up to 1 week. CaV1.2 knockdown was verified through Western blot analysis.

Brian S. Muntean and Xingjian Jin contributed equally to this work.

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TABLE 1. shRNA sequences

| Descriptions | Sequences |
|---|---|
| Scrambled control | 5′-TGACCACCCTGACCTACGGCGTGCAGTGC-3′ |
| *Cacna1c* | 5′-TCAGAAGTGCCTCACTGTTCTCGTGACCT-3′ |

Stable knockdown cell lines were obtained through puromycin selection. The following shRNA sequences were used (Table 1).

### Immunostaining studies

Cells were grown to confluence on collagen-coated glass coverslips and differentiated in serum-free media for 24 h. Cells were then fixed in 4% paraformaldehyde in PBS containing 2% sucrose, permeabilized in 10% triton X-100, incubated sequentially with primary followed by secondary antibodies for 1 h each, and finally mounted on a slide with DAPI hard set mounting media (Vector Laboratories, Burlingame, CA). The following primary antibody dilutions were used: acetylated-α-tubulin 1:10,000 (Sigma-Aldrich, St. Louis, MO) and CaV1.2 1:50 (Alomone Labs, Jerusalem, Israel). Anti-mouse Texas Red and anti-rabbit FITC fluorescent conjugated secondary antibodies were used at 1:500 (VectorLabs).

### Mitochondrial studies

MitoTracker Green FM and MitoTracker Red CMXRos (Cell Signaling Technology) were incubated with cells at 100 nM for 30 min at 37°C. MitoSOX (Life Technologies) was incubated with cells at 5 μM for 10 min at 37°C. After staining, cells were washed three times with PBS and analyzed immediately through microscopy or flow cytometry. For microscopic analysis, cells were grown on custom glass-bottom cell culture plates and imaged under a Nikon Eclipse TE2000-U microscope controlled by MetaMorph software with a 100× objective lens. For flow cytometry studies, cells were detached with trypsin, washed, and analyzed.

### DNA damage assessment

Oxidative DNA lesions were detected with an 8-oxoguanine antibody (Santa Cruz). Detached cells were fixed in 4% formaldehyde for 10 min at 37°C and permeabilized in ice-cold 90% methanol for 30 min on ice. After washing with PBS, cells were incubated in PBS containing anti-8-oxoguanine antibody (1:50), 0.5% Tween-20, and 5% FBS for 1 h. Cells were washed and incubated in PBS containing anti-mouse Texas Red antibody (1:500), 0.5% Tween-20, and 5% FBS for 1 h. Cells were then washed and analyzed with flow cytometer.

### Mitochondrial DNA and mRNA measurement

Total cellular DNA was obtained using the DNeasy Blood & Tissue Kit (Qiagen) and used for detection with PCR primers listed below to quantify the nuclear (*18S rRNA*) to mitochondrial DNA (*Coi*) ratio as described (Brown and Clayton, 2002; Bai et al., 2004). Total cellular RNA was obtained using TRIzol (Life Technologies) and reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR detection of expression genes was performed using the primers listed below comparing mitochondrial encoded oxidative phosphorylation genes (*ATP5γ1* and *CytC*) to nuclear encoded *β-Actin* as described (Yoon et al., 2010) (Table 2).

### Western blot analysis

Cells were scraped from culture plates in the presence of RIPA buffer supplemented with Complete Protease Inhibitor (Roche,

TABLE 2. Primer sequences

| Descriptions | Sequences |
|---|---|
| *Coi* F | 5′-GCCCCAGATATAGCATTCCC-3′ |
| *Coi* R | 5′-GTTCATCCTGTTCCTGCTCC-3′ |
| *18S rRNA* F | 5′-TAGAGGGACAAGTGGCGTTC-3′ |
| *18S rRNA* R | 5′-CGCTGAGCCAGTCAGTGT-3′ |
| ATP5γ1 F | 5′-AGTTGGTGTGGCTGGATCA-3′ |
| ATPγ1 R | 5′-GCTGCTTGAGAGATGGGTTC-3′ |
| CytC F | 5′-GGAGGCAAGCATAAGACTGG-3′ |
| CytC R | 5′-TCCATCAGGGTATCCTCTCC-3′ |
| *β-actin* F | 5′-TGTTACCAACTGGGACGACA-3′ |
| *β-actin* R | 5′-GGGGTGTTGAAGGTCTCAAA-3’ |

New York, NY), incubated on ice with frequent vortexing, and centrifuged. Supernatants were subjected to protein quantification and PAGE on 6–10% SDS gels followed by wet transfer to PVDF membranes and detection using β-catenin 1:1,000, CaV1.2 1:200, NF-κB p65 1:200, and GAPDH 1:1,000 (Cell Signaling Technology, Danvers, MA).

### Zebrafish

Adult wild-type AB zebrafish were obtained from the Zebrafish International Resource Center (Eugene, OR) and used for breeding. Embryos were injected with 1 mM antisense translation blocking morpholino oligos (MO; GeneTools) at the 1–2 cell stage. Zebrafish embryos were then cultured at 28.5°C in sterile egg water (Muntean et al., 2010). The following MO sequences were used: *control MO:* 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′, *cav1.2 MO:* 5′-ACA TGT TTT TGC TTT CAT TTA CCA T-3′, *pkd2 MO:* 5′-AGG ACG AAC GCG ACT GGA GCT CAT C-3′. Knockdown of CaV1.2 was verified through Western blot analysis. Briefly, zebrafish embryos were dechorionated at 28 h postfertilization and homogenized in RIPA buffer to obtain protein extracts. Western was performed on 50 μg total protein using CaV1.2 (1:200) and GAPDH (1:1,000) antibodies.

Histological examination was used to measure renal cyst formation and hydrocephalus at 3 days postfertilization. Embryos were fixed in a PBS solution containing 4% paraformaldehyde and 2% sucrose overnight at 4°C, dehydrated through an ethanol gradient, and embedded in JB4 resin (Polysciences, Inc., Warrington, PA) as specified in manufacturer's protocol. A Reichert Jung microtome was used to cut 5 μm sections which were subsequently hematoxylin and eosin stained. Heart looping was assessed at 48 h postfertilization by positioning zebrafish on their dorsal axis and recording heart beat to reveal the respective relative locations of the atrium and the ventricle.

### Data analysis

Data are reported as the mean ± standard error of the mean. All image analysis was performed using ImageJ. All flow cytometry data were analyzed with BD Accuri C6 software and were presented without any compensation gating. All data were analyzed using IBM SPSS Statistics Version 21 software by performing the student *t*-test for two group comparison or ANOVA test followed by Tukey's post-test for three or more group comparison. Statistical significance is reported with a statistical power greater than 0.8 at $P < 0.05$.

## Results

### CaV1.2 is not required for primary cilia assembly

We recently reported that the voltage gated L-type calcium channel CaV1.2 localized to primary cilia in bovine LLCPK cells (Jin et al., 2013). We performed immunostaining to verify this finding in mouse renal epithelial cells (Fig. 1). The mouse *Tg737*$^{orpk/orpk}$ cell line contains a hypomorphic mutation in an intraflagellar transport gene (*Ift88*) that is required for cells

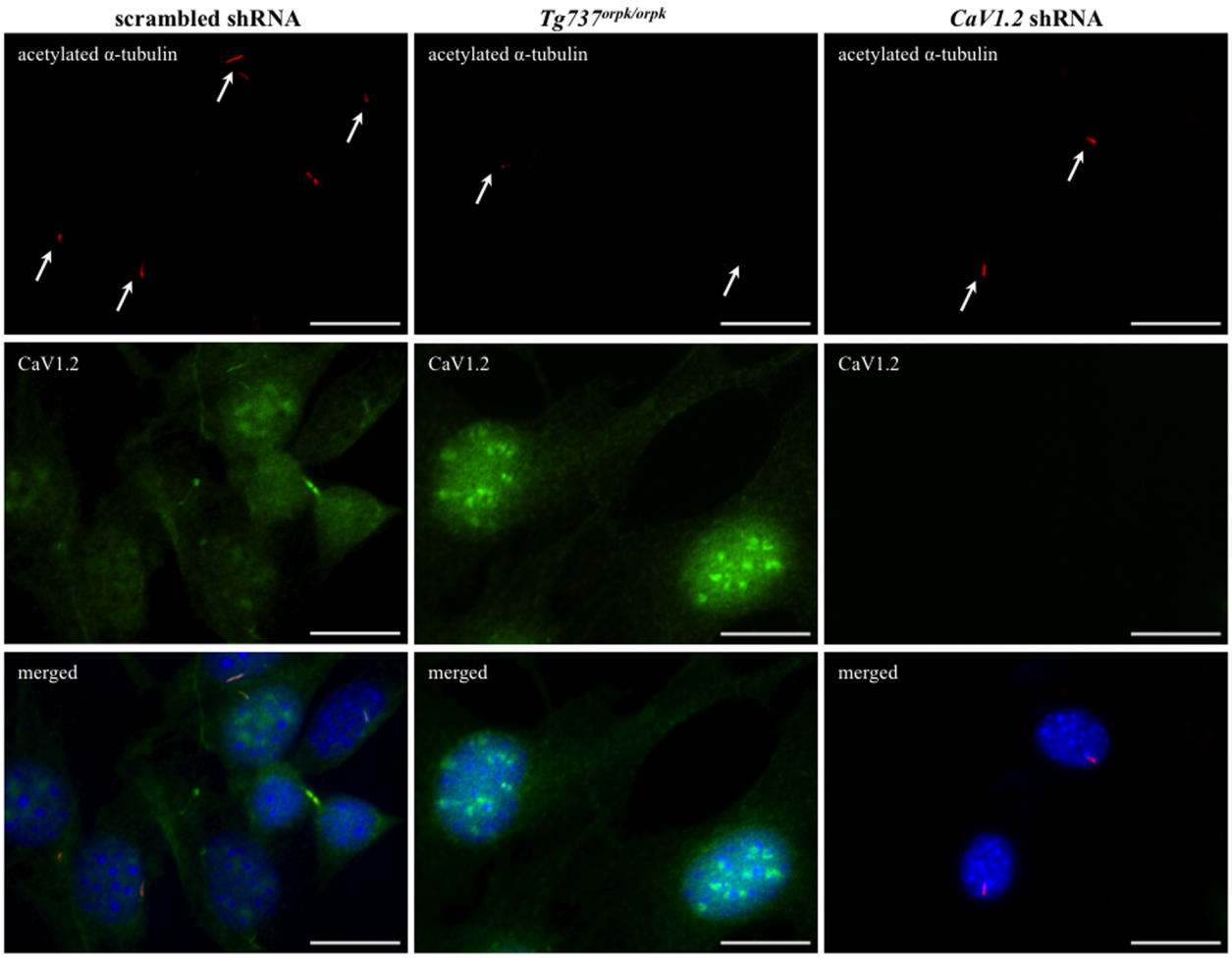


**Fig. 1. Localization of CaV1.2 to renal epithelial cilia is not required for primary cilia assembly. Immunofluorescence revealed that CaV1.2 localized to primary cilia in renal epithelial cells (scrambled shRNA) when compared with cilia-deficient cells (Tg737$^{orpk/orpk}$). The presence of primary cilia was confirmed in CaV1.2 shRNA cells. Acetylated-α-tubulin was used as a ciliary marker. Arrow indicates the presence of primary cilium, except in cilia-deficient cells. Bar = 20 μm.**

to assemble primary cilia (Moyer et al., 1994). Thus, the *Tg737*$^{orpk/orpk}$ system is a well-established model for studying cells without longer primary cilia, as verified through our immunostaining. We next asked if CaV1.2 played a role in primary cilia assembly. We generated a stable CaV1.2 shRNA knockdown mouse renal epithelial cell line and immunostaining studies revealed that primary cilia were similar to that of scrambled shRNA.

### Wnt3a induces mitochondrial biogenesis in CaV1.2-deficient but not cilia-deficient cells

Wnt signaling has recently been reported to regulate mitochondrial physiology (Yoon et al., 2010). To assess mitochondrial mass, cells were stained with Mito Tracker Green (MTG) and observed live using fluorescence microscopy. When treated with recombinant Wnt3a, mitochondrial mass increased (Fig. 2a). However, the mitochondrial mass in *Tg737*$^{orpk/orpk}$ cells was unchanged after Wnt3a treatment. We next performed this experiment in CaV1.2 shRNA cells and the results were similar to that of the scrambled control. To quantify these findings, MTG fluorescence was recorded using flow cytometry which confirmed our fluorescent observation (Fig. 2b). Our MTG studies were further validated using a common technique by comparing mitochondrial DNA (*Coi*) to nuclear DNA (*18S rRNA*) (Brown and Clayton, 2002; Bai et al., 2004). As expected, Wnt3a did indeed statistically increase mitochondrial biogenesis in scrambled and CaV1.2 shRNA but not in *Tg737*$^{orpk/orpk}$ cells (Fig. 2c). Our immunofluorescence study showed that Wnt3a did not alter CaV1.2 localization to cilia (Table 3).

### Wnt3a increases mitochondrial activity in CaV1.2-deficient cells while decreasing activity in cilia-deficient cells

We next asked if Wnt3a would have an effect on mitochondrial oxidative phosphorylation (activity) in *Tg737*$^{orpk/orpk}$ cells. Similar to before, we stained cells with Mito Tracker Red (MTR). Unlike MTG, MTR staining is dependent on the mitochondrial membrane potential (Poot and Pierce, 2001; Pendergrass et al., 2004). Therefore, increased staining

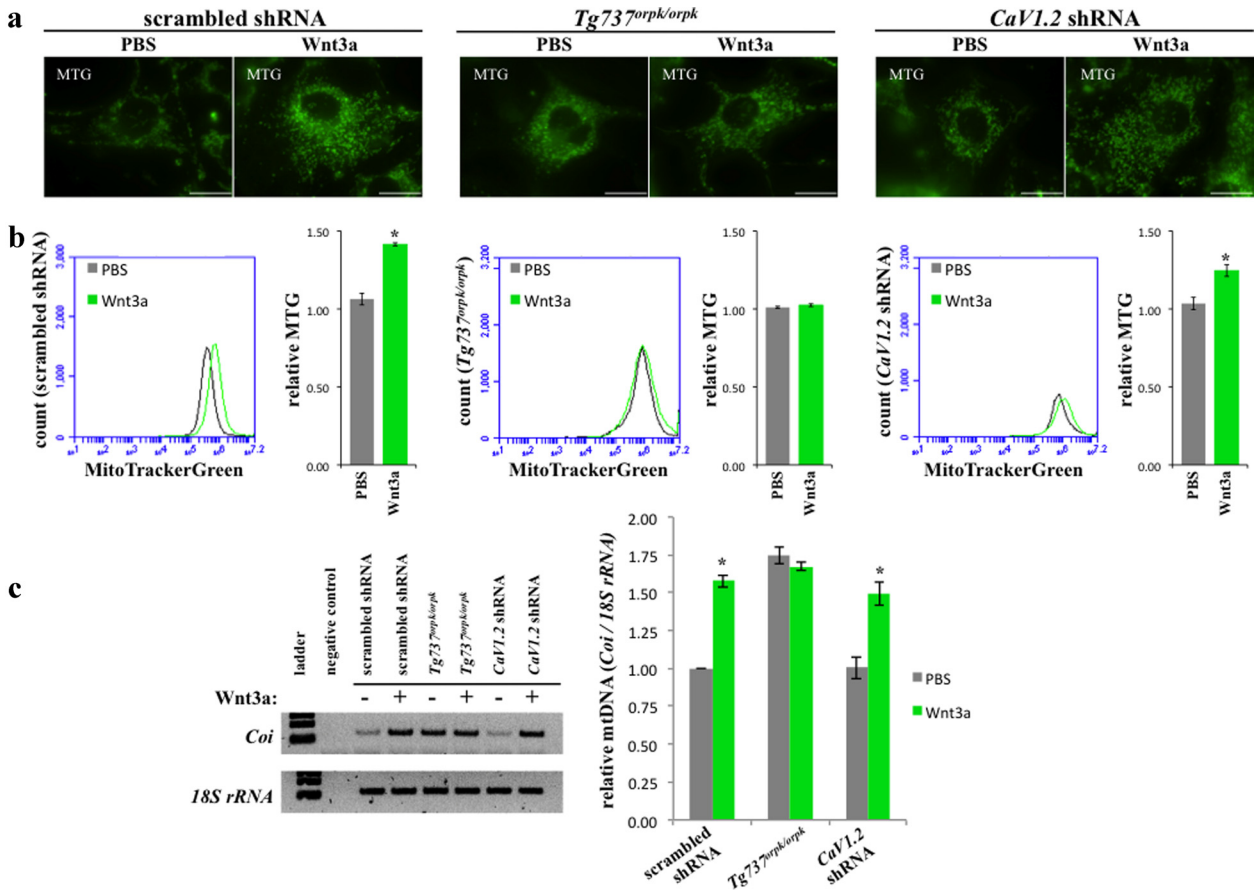


**Fig. 2. Wnt3a induces mitochondrial biogenesis in CaV1.2 shRNA but not Tg737$^{orpk/orpk}$ cells. a: Mitochondrial mass was assessed by staining cells with Mito Tracker Green. Wnt3a was found to induce mitochondrial mass in scrambled and CaV1.2 shRNA cells but had no effect on Tg737$^{orpk/orpk}$ when examined using fluorescence microscopy (bar = 20 μm). b: Results were quantified using flow cytometry. c: Mitochondrial DNA was measured using PCR by taking the ratio of a mitochondrial gene (Coi) to a nuclear gene (18S rRNA) (N = 3).**

correlates to increased oxidative phosphorylation. As expected, Wnt3a increased MTR staining in scrambled and CaV1.2 shRNA cells when observed using fluorescence microscopy (Fig. 3a). However, mitochondrial activity decreased in *Tg737*$^{orpk/orpk}$ cells. We again quantified our findings using flow cytometry (Fig. 3b). Wnt3a significantly increased mitochondrial activity in scrambled and CaV1.2 shRNA while significantly decreasing activity in *Tg737*$^{orpk/orpk}$ cells. To verify these results, we compared expression of two key mitochondrial encoded oxidative phosphorylation genes (ATP Synthase 5γ1 and Cytochrome *c*) relative to that of nuclear encoded β-actin (Fig. 3c).

TABLE 3. CaV1.2 ciliary localization

| | % CaV1.2 localization to cilia | N |
|---|---|---|
| PBS (control) | | |
| Scramble shRNA | 91.1 | 45 |
| *Tg737*$^{orpk/orpk}$ | 91.7 | 36 |
| *CaV1.2* shRNA | 0.0 | 41 |
| Wnt3a (100 ng/ml) | | |
| Scramble shRNA | 90.4 | 52 |
| *Tg737*$^{orpk/orpk}$ | 92.3 | 39 |
| *CaV1.2* shRNA | 0.0 | 46 |

### Wnt3a increases ROS and DNA damage in CaV1.2-deficient but not in cilia-deficient cells

An inevitable consequence of oxidative phosphorylation is the generation of reactive oxygen species (ROS) (Boveris et al., 1972; Boveris and Chance, 1973). MitoSOX is a cell permeable red fluorescent indicator specific for mitochondrial ROS. We therefore stained cells with MitoSOX and observed a significant increase in mitochondrial ROS in scrambled and CaV1.2 shRNA after treatment with Wnt3a (Fig. 4a). A significant decrease in staining was observed in *Tg737*$^{orpk/orpk}$ cells (Fig. 4b). Genomic DNA can be damaged by ROS to form DNA lesions resulting from mismatched repairs (Kasai et al., 1984). Thus, we quantified the levels of 8-Oxoguanine, a common DNA lesion formed by mismatched Adenine (Kasai, 1997). Treatment with Wnt3a was found to increase 8-Oxoguanine in scrambled and CaV1.2 shRNA while no change was observed in *Tg737*$^{orpk/orpk}$ cells (Fig. 4c).

### Cilia modulates Wnt signaling to regulate CaV1.2 expression

As previously reported, Wnt3a treatment induced β-catenin expression in all cells (Aberle et al., 1997). We confirmed this in our system, including in *Tg737*$^{orpk/orpk}$ and CaV1.2 shRNA cells (Fig. 5). Consistent with previous study (Corbit et al., 2008),

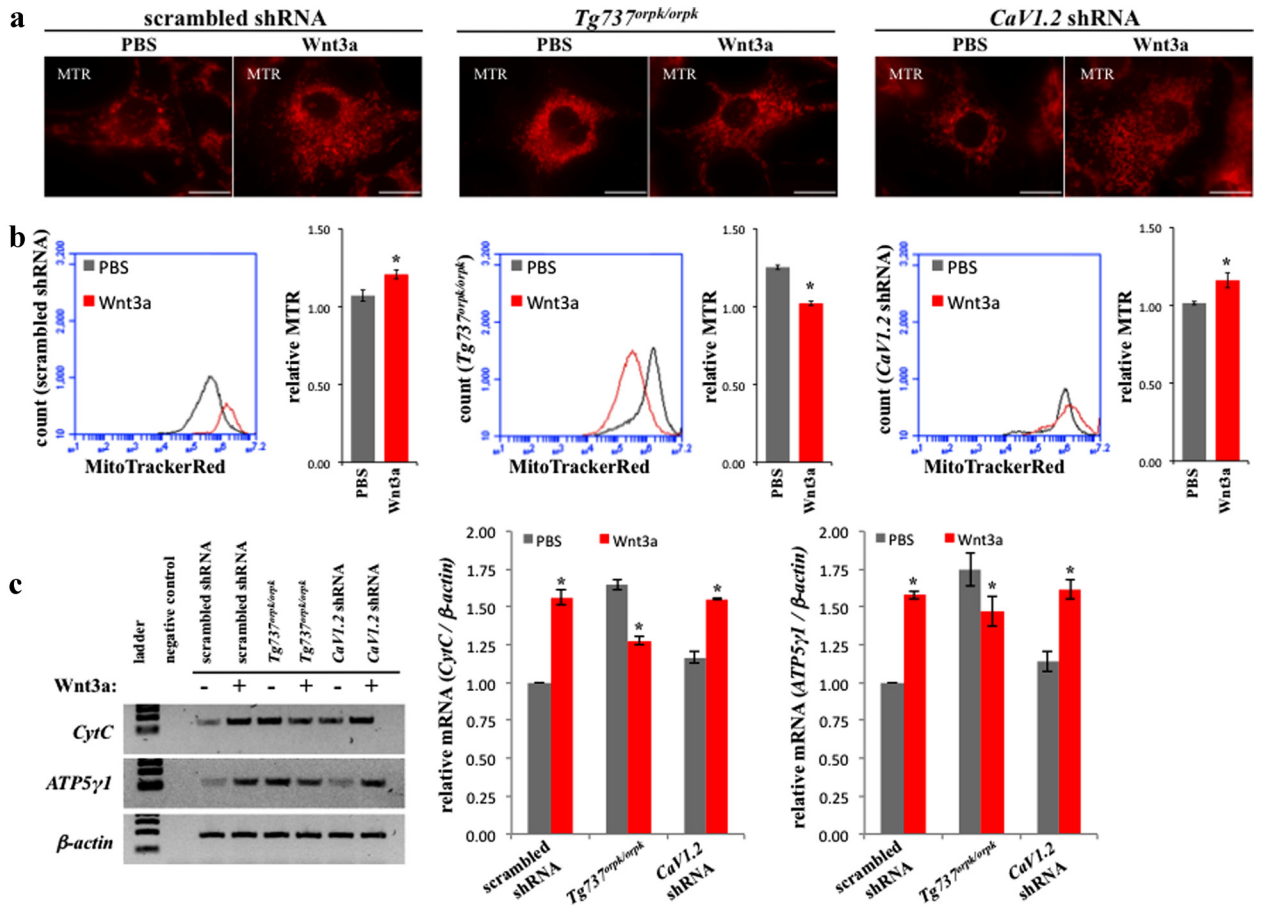


**Fig. 3. Wnt3a increases mitochondrial activity in CaV1.2 shRNA but decreasing activity in Tg737$^{orpk/orpk}$ cells. a: Mitochondrial oxidative phosphorylation was used to indicate activity through Mito Tracker Red staining. Wnt3a was found to increase oxidative phosphorylation in scrambled and CaV1.2 shRNA cells while decreasing oxidative phosphorylation in Tg737$^{orpk/orpk}$ when examined using fluorescence microscopy (bar = 20 μm). b: Results were quantified using flow cytometry. c: Two mitochondrial mRNAs encoded oxidative phosphorylation genes (CytC and ATP5γ1) were measured using PCR normalized to nuclear encoded β-actin (N = 3).**

*Tg737$^{orpk/orpk}$* cells showed a higher basal level of β-catenin than control. Further, Wnt3a treatment increased CaV1.2 expression in scrambled shRNA while decreasing CaV1.2 in *Tg737$^{orpk/orpk}$* cells. Of note is that CaV1.2 expression was not detectable in CaV1.2 shRNA cells, confirming knockdown of CaV1.2 in our stable cell line.

The DNA damage response (DDR) is a cellular mechanism to recover from DNA lesions, such as 8-Oxoguanine (Kasai et al., 1984; Jackson and Bartek, 2009). One arm of this cell survival pathway is the activation of nuclear factor κB p65 (NFκB p65) (Janssens and Tschopp, 2006). Through Western blot analysis, we also found that Wnt3a induced NFκB p65 expression in scrambled and CaV1.2 shRNA (Fig. 5). On the other hand, *Tg737$^{orpk/orpk}$* cells expressed a high basal level of NFκB p65 which decreased in response to Wnt3a. In addition, CaV1.2 expression was found to correlate with NFκB p65.

### CaV1.2 knockdown zebrafish develop PKD phenotypes

We have shown that CaV1.2 localizes to primary cilia and have now elucidated the mechanism by which CaV1.2 expression is regulated in renal epithelial cells. To assess the biological significance of CaV1.2 expression, we used antisense morpholinos to knockdown CaV1.2 in zebrafish. Knockdown of the ciliary calcium channel polycystin-2 in zebrafish has been reported to result in PKD phenotypes including renal cyst formation, hydrocephalus, and left-right asymmetry (Obara et al., 2006). Our study showed that knockdown of *pkd2* increased CaV1.2 expression (Fig. 6). This slight increase in CaV1.2 was significant compared to the control morpholino. Interestingly, similar phenotypes were observed in CaV1.2 morpholino (*cav1.2 MO*) zebrafish. Compared with a non-specific control morpholino (*control MO*) injection, *cav1.2 MO* zebrafish developed renal cysts (Fig. 7a), hydrocephalus (Fig. 7b) and various heart-looping defects (Fig. 7c). As generally accepted (Bakkers, 2011), left-right asymmetry was assessed by measuring the relative position of the cardiac atrium and ventricle with respect to the dorsal axis (Supplemental Movie 1).

## Discussion

Non-motile primary cilia have been found to play a critical role in Wnt signaling by restricting β-catenin accumulation. Overexpression of polycystin-1 (a ciliary signaling receptor) inhibits GSK3β and stabilizes β- catenin (Kim et al., 1999).

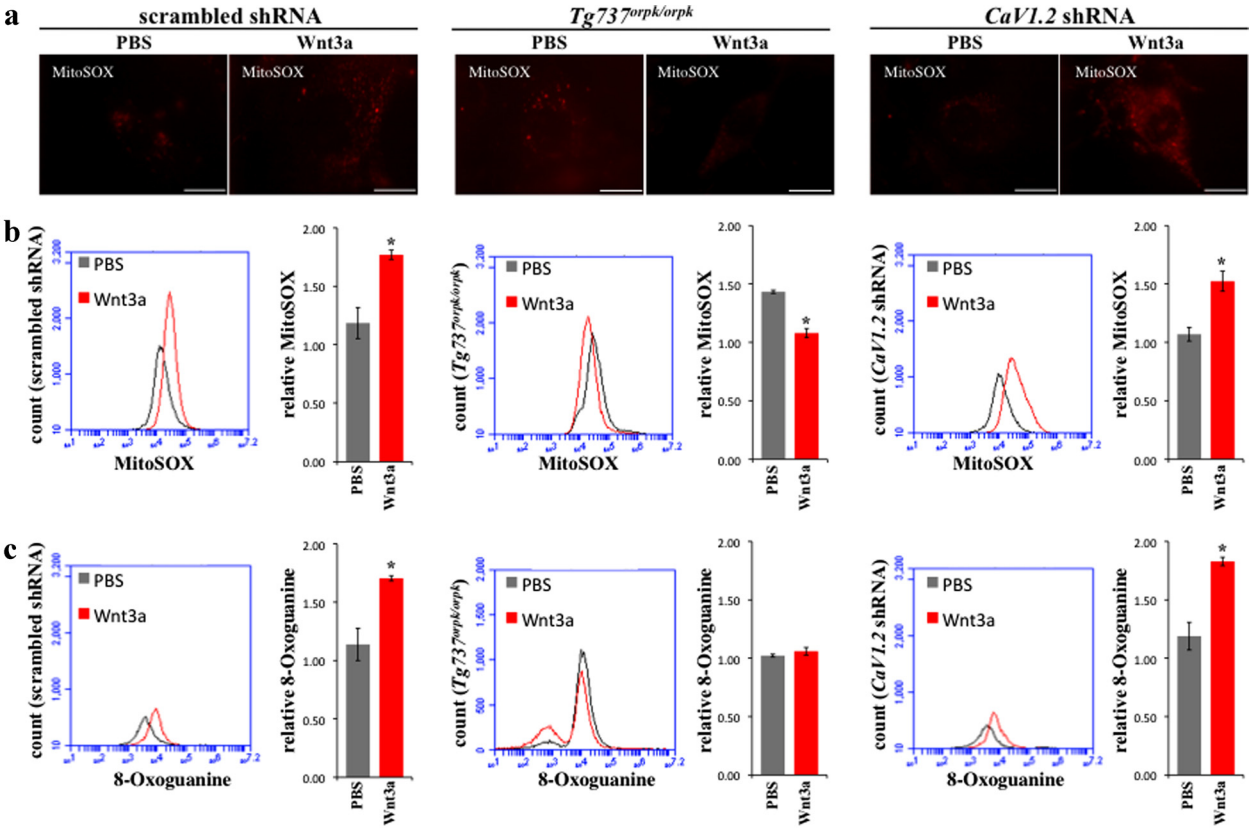


**Fig. 4. Wnt3a increases ROS and DNA damage in CaV1.2 shRNA but not in Tg737$^{orpk/orpk}$ cells. a: Mitochondrial ROS was assessed by staining cells with MitoSOX. Wnt3a was found to increase ROS in scrambled and CaV1.2 shRNA cells while decreasing ROS in Tg737$^{orpk/orpk}$ when examined using fluorescence microscopy (bar = 20 μm). b: Results were quantified using flow cytometry. c: Wnt3a increased formation of the oxidative DNA lesion 8-Oxoguanine in scrambled and CaV1.2 shRNA cells but had no effect on Tg737$^{orpk/orpk}$ cells (N = 3).**

Polycystin-2 (encoded by *Pkd2*) is calcium channel forming protein found in primary cilia. In *Pkd2*$^{-/-}$ embryos, cilia length was found to be decreased while β-catenin was upregulated (Kim et al., 2009). Interestingly, transgenic mice overexpressing β-catenin also developed cystic kidneys (Saadi-Kheddouci et al., 2001). Further, LRP6$^{-/-}$ (a component of the Wnt receptor complex) mouse embryos die in utero with cystic kidneys (Pinson et al., 2000). Thus, primary cilia and Wnt signaling play a crucial role in PKD (Corbit et al., 2008).

Given that Wnt signaling also modulates mitochondrial physiology (Yoon et al., 2010), we examined the role of cilia in regards to mitochondria. *Tg737*$^{orpk/orpk}$ contains an intron insertion at the 3′ end of the intraflagellar transport 88 (*Ift88*) gene which results in a hypomorphic mutation that prevents ciliogenesis (Moyer et al., 1994). We used *Tg737*$^{orpk/orpk}$ cells as a model for a cilia-deficient system. Through immunostaining, we confirmed the absence of cilia in *Tg737*$^{orpk/orpk}$ cells compared with control (Fig. 1). The voltage-gated L-type calcium channel CaV1.2 also localized to primary cilia. We generated a stable CaV1.2 shRNA cell line and observed no changes in primary cilia compared with control. Further, treatment with Wnt3a had no effect on cilia number or length in scrambled or CaV1.2 shRNA cells (data not shown). Thus, CaV1.2 does not seem to play a role in ciliogenesis.

Mitochondrial biogenesis, oxidative phosphorylation, and generation of reactive oxidative species (ROS) were increased in response to Wnt3a in control renal epithelial cells (Figures 2–4). The elevated levels of oxidative stress increased the formation of DNA lesions and the cellular DNA damage response (DDR). An interesting aspect of this response was an increase in expression of CaV1.2 (Fig. 5). In CaV1.2 knockdown cells, Wnt3a induced a similar effect on mitochondria and DDR. This data suggests that CaV1.2 is a downstream effector in regard to Wnt signaling. In cilia-deficient cells, Wnt3a was unable to induce mitochondrial biogenesis and decreased mitochondrial activity, ROS production, and DDR. CaV1.2 was found to be overexpressed in cilia-deficient cells as a compensatory mechanism; however, its expression decreased following Wnt3a treatment. Therefore, cilia length plays a role in regulating CaV1.2 expression through modulation of Wnt signaling.

Defective primary cilia, indicated by either depletion of key ciliary proteins or fundamental changes in structure/length, results in PKD phenotypes (Wilson, 2004). Here we show that CaV1.2 is a biologically significant ciliary protein. In the absence of CaV1.2 in zebrafish (Fig. 6), PKD phenotypes including renal cyst formation, hydrocephalus, and left-right asymmetry defects were observed (Fig. 7). Moreover, CaV1.2 was found to be overexpressed in *pkd2* knockdown zebrafish. This is intriguing given that both CaV1.2 and PC2 are calcium channel forming proteins in the primary cilium, which further suggests a role for CaV1.2 in PKD pathogenesis. Therefore, CaV1.2 not only localizes to renal epithelial primary cilia, but it is also required for cilia function.

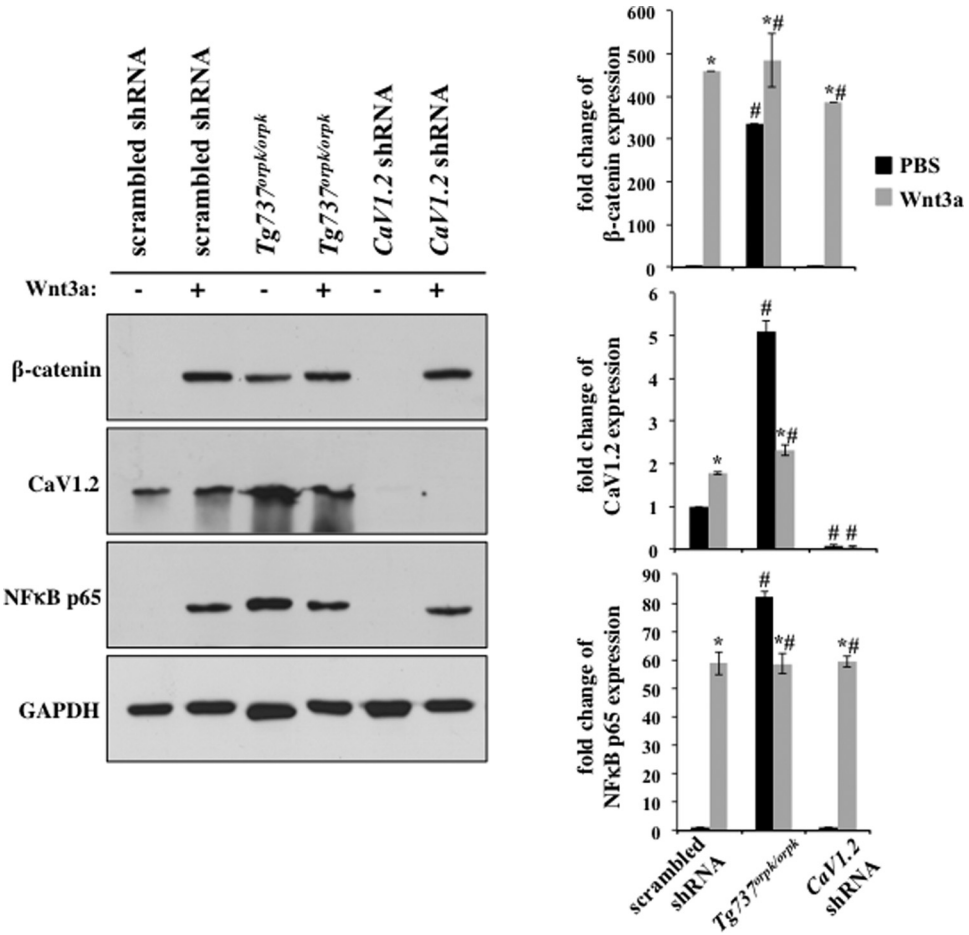


**Fig. 5. Cilia modulates Wnt signaling to regulate CaV1.2 expression. Western blotting was performed on cellular protein extracts. Wnt3a treatment increased β-catenin accumulation in all cells, however, Tg737$^{orpk/orpk}$ cells displayed high basal levels of β-catenin. NF-κB p65 was blotted as a readout for DNA damage response (DDR) to ROS induced DNA lesions. Wnt3a induced NF-κB p65 expression in scrambled and CaV1.2 shRNA cells but decreased NF-κB p65 in Tg737$^{orpk/orpk}$ cells. CaV1.2 was overexpressed in Tg737$^{orpk/orpk}$ cells. Wnt3a treatment induced CaV1.2 expression in scrambled shRNA while decreasing expression in Tg737$^{orpk/orpk}$ cells. Data normalized to GAPDH for analysis (N = 3). Asterisks indicate significant difference from the corresponding control group ($P < 0.05$). # signs denote significant difference from the corresponding scrambled shRNA group ($P < 0.05$).**

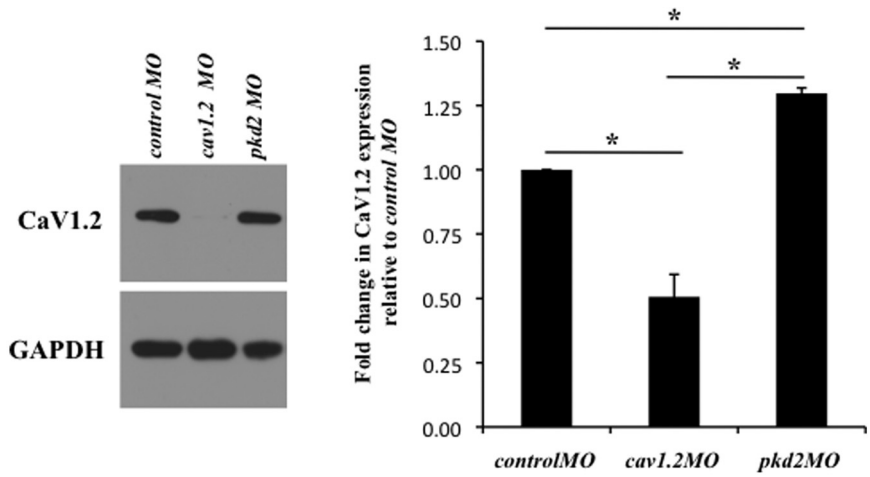


**Fig. 6. pkd2 knockdown increases CaV1.2 expression in zebrafish. Embryonic zebrafish protein extracts were obtained at 28 h postfertilization. Western blotting showed that cav1.2MO effectively reduced CaV1.2 expression compared with controlMO. pkd2MO zebrafish were used to further verify morpholino specificity. Results were quantified through one-way ANOVA with Tukey post-test. Statistical significance is reported with a mean difference at the 0.05 level and denoted with an asterisk (N = 3).**

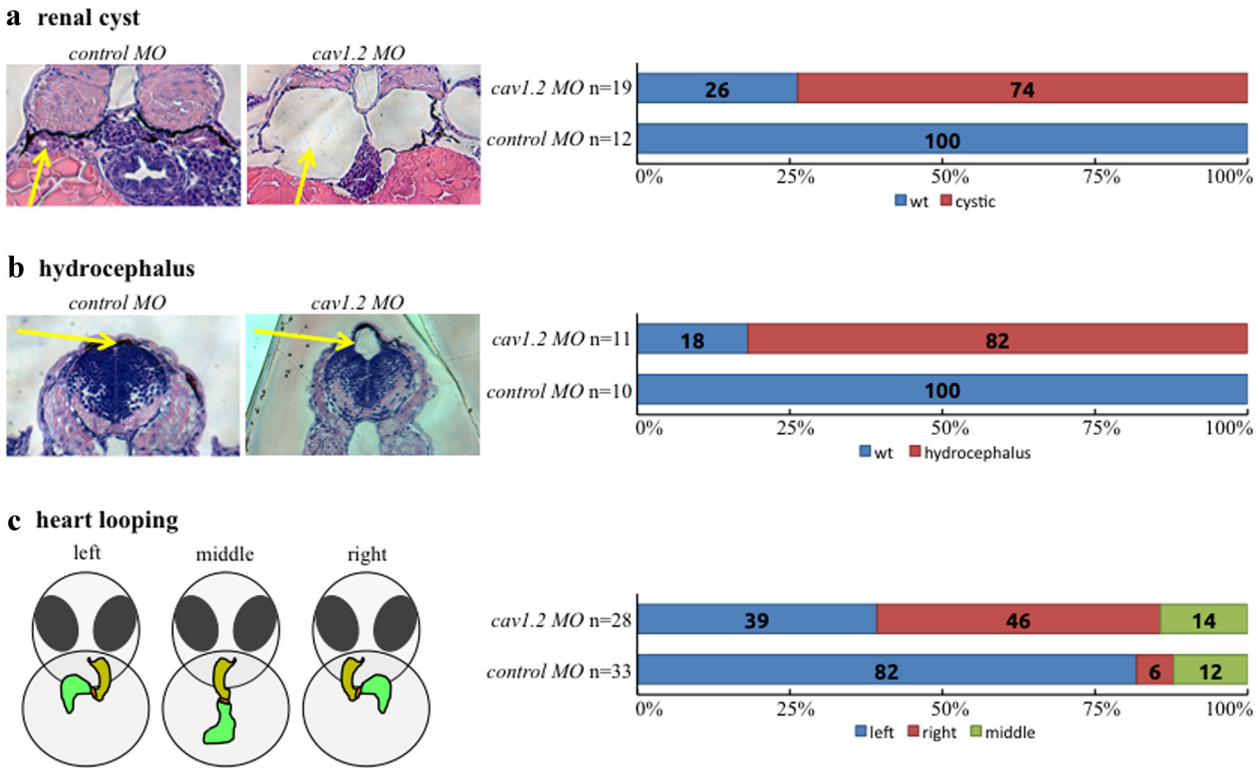


**Fig. 7. CaV1.2 knockdown zebrafish develop renal cysts, hydrocephalus, and left-right asymmetry defects. CaV1.2 was knocked down in zebrafish (cav1.2 MO) using morpholino microinjection and compared to a scrambled control morpholino (control MO) to assess phenotypes. Renal cyst formation (a) and hydrocephalus (b) were measured at 3 days postfertilization through standard H&E staining as indicated by arrows. Left-right asymmetry was determined by measuring heart looping (c) at 2 days postfertilization under live microscopy.**

Although it has been known that abnormal Wnt signaling leads to renal cyst formation (Lancaster et al., 2009), the underlying mechanism has been unclear. One proposed explanation is that Wnt signaling regulates renal cell proliferation and planar cell polarity to maintain renal tubule homeostasis (Happe et al., 2009). Our present study further suggests that cilia regulate Wnt signaling which ultimately controls CaV1.2 expression. Therefore, in the absence of CaV1.2, ciliary function is compromised leading to formation of renal cysts. In addition, our study also shows a previously unrecognized relationship between primary cilia and mitochondrial function. Overall, we propose that Wnt3a induces β-catenin accumulation in a cilia-dependent manner. This in turn increases mitochondrial biogenesis, oxidative phosphorylation, and generation of ROS that cause genomic DNA damage. The DDR triggers NFκB p65 expression ultimately resulting in increased CaV1.2 expression.

## Acknowledgments

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# Vascular Endothelial Primary Cilia: Mechanosensation and Hypertension

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**Abstract:** Primary cilia are sensory organelles that extend from the cell surface and sense extracellular signals. Endothelial primary cilia protruding from the inner surface of blood vessel walls sense changes in blood flow and convert this mechanosensation into an intracellular biochemical/ molecular signal, which triggers a cellular response. Primary endothelial cilia dysfunction may contribute to the impairment of this response and thus be directly implicated in the development of vascular abnormalities such as hypertension and aneurysms. Using both *in vitro* techniques as well as *in vivo* animal models, we and others have investigated fluid flow mechanosensory functions of endothelial cilia in cultured cells, animal models and autosomal dominant polycystic kidney disease (ADPKD) patients. More in-depth studies directed at identification of the mechanisms of fluid flow sensing will further enhance our knowledge of cilia-dependent vascular pathology. Although the current treatments aimed at treating the cardiovascular symptoms in ADPKD patients successfully slowed the progression of cyst growth, there is growing evidence which suggests that drugs which interfere with primary cilia function or structure could reduce cardiovascular complications in ADPKD. This review is to summarize the most recent studies on primary endothelial cilia function in the vascular system and to present primary cilia as a novel therapeutic target for vascular hypertension.

**Keywords:** Cardiovascular, cell division, fluid-shear, hypertension, primary cilia.

## INTRODUCTION

It has been over a century since primary cilia have been visualized, but the study of their sensory role is, comparatively, a new field [1]. Like other cellular organelles i.e. nucleus, Golgi bodies, mitochondria etc. cilia can be considered as a separate entity and have the following specialized functions:

- Mechanosensation [2-4].
- Shear-stress sensation [5-7].
- Nodal flow generation [8-10].
- Nodal flow sensation [11, 12].
- Osmosensation [13-15].
- Chemosensation [16-19].
- Light sensation [20-22].
- Smell sensation [23, 24].
- Gravitational sensation [25].
- Developmental regulation [26-28].
- Fluid clearance [29, 30].
- Sperm motility [31-33].
- Fluid transportation [34-36].
- Oocyte transportation [37, 38].
- Sound-wave sensation [39-41].

In order to study a cilium, it must be divided into five distinct structural blocks – the ciliary membrane, the soluble compartment, the axoneme, the ciliary tip and the basal body complex (Fig. **1a**). The primary ciliary membrane has a distinct lipid bilayer composition and is continuous with the cell membrane at the transition fiber. It houses various membrane mechanosensory receptors, ligand-activated chemoreceptors and ion channels to support mechanosensation, chemosensation and ion influx in response to extracellular stimuli. The soluble or matrix compartment also called the cilioplasm is composed of fluid materials to support signaling activities. The axoneme, which emanates from the basal body, is composed of nine pairs of microtubules forming heterodimer structure. Apart from delivering cellular components in and out of the ciliary shaft, it plays a significant role in maintaining a long ciliary structure. The ciliary tip contains specialized proteins, but

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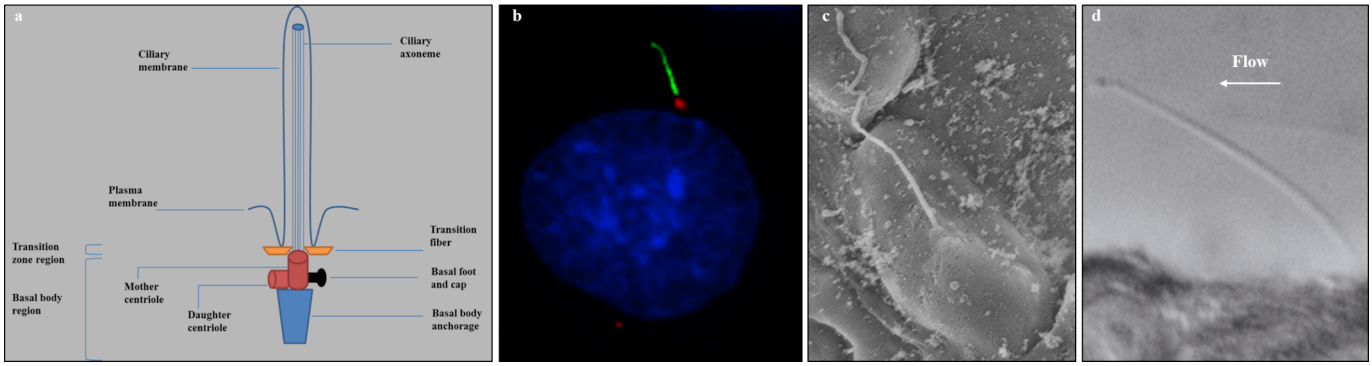


**Fig. (1).** Mechanosensory cilium. A primary cilium is a cellular organelle extending from the apical surface of many cell types. The mechanosensory cilia act as antennas to receive and transmit extracellular fluid-shear stress into cellular biochemical signals. **(a)** A cilium is composed of a ciliary membrane, a soluble matrix, a ciliary axoneme, and a basal body complex. The *ciliary membrane* contains various sensory proteins. The *soluble matrix* contains many regulatory proteins that are involved in signal transduction networks. The *ciliary axoneme* is surrounded by the nine pairs of microtubules that are thought to control the length of a cilium. The *basal body complex* is the anchoring point for axonemal microtubules. (**b**) Primary cilia found on the surface of endothelial cells can be localized by a simple immunostaining experiment using antibody against acetylated α-tubulin (green) to label primary cilia and pericentrin (red) to label centriole or basal body. The nucleus is counterstained with DAPI to label DNA. (**c**) Mouse primary epithelial cilia can be studied *in vivo* by scanning electron microscopy (SEM) to reveal kidney epithelial cilia protruding from the surface of kidney epithelial cells towards the lumen of kidney tubules. (**d**) Primary cilia are sensory organelles that sense fluid-shear stress on the apical surface of cells. Fluid flow that produces enough drag-force on the cells will bend sensory cilia. Part of this figure is reproduced from [152].

their roles are still unknown. The basal body complex is composed of the mother and daughter centrioles connected by rootlets and emanate from the basal body anchorage. The mother centriole is the basal body from which the cilium protrudes [42].

Primary Cilia is a term commonly used to denote "9+0" non-motile cilia. These have nine parallel doublet microtubules (denoted by '9') and a central pair of microtubule is absent within the central sheath (denoted by '0'). Length of a primary cilium can vary from 2-50 μM and it has a diameter of approximately 0.25 μM [42]. Primary cilia are found on several mammalian cell types i.e. endothelia (Fig. **1b**) [43-46], epithelia (Fig. **1c**) [47-49], neurons [50-53], osteoblasts [54-56], fibroblasts and others [57-59]. Thus, abnormal ciliary proteins and/or abnormal ciliary function can be associated with various pathological conditions collectively known as ciliopathies; which include polycystic kidney disease (PKD), nephronophthisis, bardet biedl syndrome, left-right asymmetry defect, oral facial syndrome, obesity, hypertension and others [60]. Primary cilia can be activated by bending through perfusing cells with fluid-shear stress (Fig. **1d**) or by mechanical stress [61]. Bending cilia by apical fluid perfusion with physiologically relevant flow rate [2, 62] or by suction through a micropipette [63] can activate primary cilia. Consequently, mechanosensory ciliary proteins such as polycystin-1 [5], polycystin-2 [7], transient receptor potential-4 [64], fibrocystin [65], and probably many others convert this mechanical force into an intracellular biochemical signal [66, 67].

## PRIMARY ENDOTHELIAL CILIA AS BLOOD PRESSURE SENSORS

The control of the circulatory system depends largely on the mechanical properties of blood vessels in both normal and pathological conditions. The continuous change in blood vessel diameter triggered by contraction and relaxation of vascular smooth muscle cells is important for normal blood flow [68-71].

Different blood vessels are subjected to changes in blood pressure as well as changes in the hemodynamic forces which induce distinct responses within the vascular tree and allows for tissue perfusion. Resistant arteries have the most influence on blood flow and they are subjected to the continuous effect of blood flow and pressure. An increase in blood pressure induces a vasoconstriction known as the myogenic tone, which facilitates the action of the sympathetic nervous system [72]. When transmural blood pressure increases, vessel diameter is reduced [73-75]; while faster flow velocity (shear stress) increases blood vessel diameter [74, 76, 77]. Vascular endothelial cells lining the inner wall of blood vessels can sense changes in blood flow and pressure and convert these mechanical changes into changes of smooth muscle tone [74, 78].

The pulsatile nature of blood flow through the vasculature exerts different types of mechanical forces such as shear stress, cyclic strains and hydrostatic pressure that can impact the physiology of the blood vessel wall. Being the inner most layer and in direct contact with blood flow, the endothelial cell layer bears the most frictional forces induced by the flow of blood. Though these forces are practically impossible to differentiate *in vivo*, they can be distinguished from one another in *in vitro* and *ex vivo* studies [79-81]. In this review, we will focus on shear stress which is the most studied mechanical force in cilia research.

We have previously demonstrated the presence as well as the function of endothelial primary cilia as fluid mechanosensor *in vitro* in mouse aortic endothelial cells, *ex*

*vivo* in isolated mouse arteries and *in vivo* in mouse models as well as in blood vessels from human patients [5, 7]. The presence as well as the size of primary endothelial cilia varies with respect to the level of fluid shear stress or fluid turbulence. For example, blood vessels with relatively low fluid shear stress have longer cilia while blood vessels with high fluid shear stress are devoid of cilia or have shorter cilia simply due to the inability of cilia to tolerate high levels of shear stress, which could induce its disassembly. Consequently, the ciliary mechanosensory function in those areas of high fluid shear stress could be minimized or substituted by other vessel components such as the glycocalyx [82]. Sensory proteins localized to primary endothelial cilia such as polycystins can detect any sudden increase in blood pressure or shear stress inside the blood vessel [5, 7]. This in turn, leads to the influx of calcium ions to the cilioplasm followed by an increase in intracellular calcium concentration. As a result, primary cilia enable cells to translate this extracellular fluid mechanics into a complex intracellular signaling cascade. This signaling cascade eventually leads to the activation of endothelial nitric oxide synthase (eNOS), an endothelial enzyme that synthesizes nitric oxide gas. Nitric oxide diffuses from endothelial cells to the surrounding smooth muscle cells and promotes vasodilation [83-85]. Both polycystin-1 and polycystin-2, an 11 transmembrane protein with a long extracellular domain and a cation channel with 6 transmembrane domains, respectively are expressed in the endothelial and vascular smooth muscle cells of all major blood vessels [86]. We previously reported the loss of response to fluid-shear stress in mouse endothelial cells with *knockdown* or *knockout* of *PKD2* [7]. In addition to the mouse data, polycystin-2 null endothelial cells generated from *PKD2* patients that do not show polycystin-2 in the cilia are unable to sense fluid flow. Therefore, mutations in both *PKD1* and *PKD2* have been shown to contribute to hypertension [87], in part by the failure to convert an increase in mechanical blood flow into cellular NO biosynthesis [5, 7]. In summary, by sensing any increase in fluid shear stress, primary cilia can activate different cellular mechanisms in order to lower total peripheral resistance and consequently, blood pressure.

It is crucial to discern the physiological mechanism behind mechanosensory role of primary cilia in order to clearly understand the correlation between primary cilia abnormalities and cardiovascular pathology and in particular hypertension. A new mechanism has been proposed that involves polycystin-1 and polycystin-2 [5, 7]. Endothelial cells without primary cilia were isolated from $Tg737^{Orpk/Orpk}$ mice to examine the mechanosensory role of primary cilia. In addition and to confirm the role of polycystin-1 and polycystin-2 as mechanosensory proteins, endothelial cells were collected from PKD mice and ADPKD human patients. Polycystins are absent in the cilia of these cells and present in *Tg737* mice cells, but localized at the base of the "stubby" primary cilia. Different magnitudes of fluid shear stress were applied to these cells. Though shear induced cytosolic calcium was increased in normal endothelial cells, diseased artery and mutant endothelial cells did not show any calcium response to fluid shear stress [5, 7]. To verify cilia function specifically against fluid shear stress, a new technique involving artery perfusion in glass capillary was utilized [7, 88]. It was observed that though cilia react normally when other mechanical stimuli are applied, response to fluid shear stress is highly altered.

To dissect the molecular mechanisms downstream of cilia function, different biochemical and pharmacological inhibitors were used in these studies to block downstream molecular targets in endothelial cells. When EGTA is used to block extracellular calcium, both cytosolic calcium and nitric oxide production was inhibited. In addition, L-NAME ($N^G$-nitro-L-arginine methylester), an eNOS inhibitor, was found to block shear induced nitric oxide synthesis but not cytosolic calcium increase. This indicates the necessity of extracellular calcium influx for the downstream generation of nitric oxide. To examine calcium dependent mechanism of nitric oxide biosysnthesis, calphostin C, a PKC inhibitor and W7, a calmodulin antagonist were employed. Results showed that PKC and calmodulin function downstream of calcium pathway and blocking either of these will inhibit nitric oxide synthesis. When pharmacological blockers Akt inhibitor II, LY-294,002, and wortmannin were applied, role of Akt was confirmed in shear induced nitric oxide production. These studies in addition to others demonstrate an interaction between mechanosensory polycystin-1 and poylycystin-2 and that absence of this interaction results in disturbance of localization of polycystin-2 to primary cilia and hence, disturb ciliary functions. In summary, these studies lead us to propose that, after sensing fluid shear stress, polycystin-1 interacts with and activates polycystin-2, a calcium channel which induces an intracellular calcium influx. This is followed by activation of PKC and formation of calcium-calmodulin complex. Our data also indicate that Akt/PKB is also involved in regulation of eNOS activation and the generation of nitric oxide in response to fluid shear (Fig. **2a** and **b**).

## ROLE OF PRIMARY CILIA IN THE CARDIO-VASCULAR SYSTEM

Despite its well-known and demonstrated function as a mechanosensory organelle in the cardiovascular system, the role of primary cilia in this system is still a widely debated topic. Recent studies have successfully demonstrated the presence of primary cilia throughout the cardiovascular system. Primary cilia have been observed in endocardia [6, 89], arterial endothelia [2, 7], venous endothelia [90], corneal endothelia [44, 46], arterial smooth muscle cells [91] and airway smooth muscle cells [92].

### Primary Cilia and Heart Development

Recent studies have demonstrated an important role of cardiac primary cilia in harmonizing heart development, and defects in these cilia are associated with inherited heart disease. Different types of cilia are present in different chambers throughout heart development to control cardiogenesis. For example, sensory nodal cilia play an important role in regulating signaling mechanisms essential for the establishment of left-right asymmetry, a process for regulating heart morphogenesis. In addition, primary cilia are present on the surface of cardiomyocytes during heart development and houses different receptor complexes which coordinate various signaling mechanisms that are important

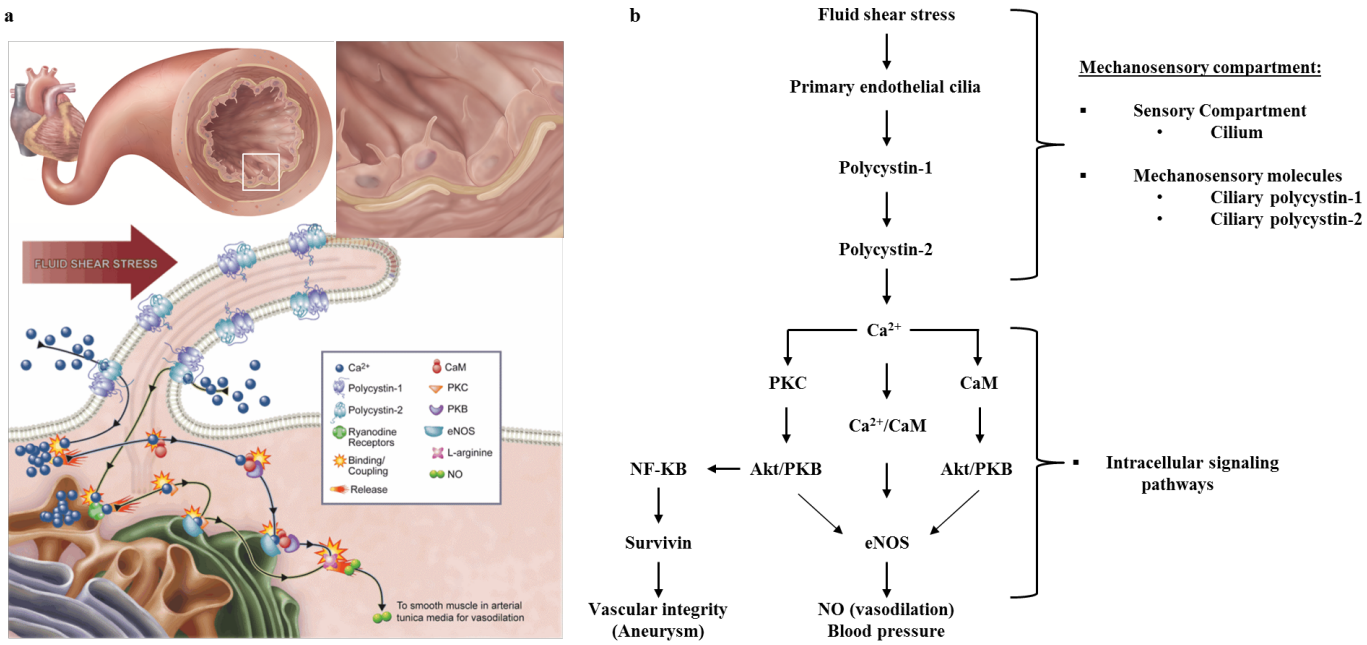


**Fig. (2).** Endothelial primary cilia as regulators of blood pressure through nitric oxide production and vascular architecture through survivin. (**a** and **b**) Nitric oxide synthesis and survivin expression depend on the presence of functional sensory cilia housing sensory complex, polycystin-1 and polycystin-2. Bending of primary endothelial cilia by fluid flow activates polycystin complex and initiates a series of intracellular signaling cascades resulting in the synthesis and release of NO gas, which vasodilates smooth muscles in the vasculature. These intracellular signaling cascades includes calcium influx, calcium-calmodulin, protein kinase C, Akt/PKB and eNOS. Survivin downregulation is involved in aneurysm formation. Fluid shear stress induced the activation of polycystin-1/2 complex followed by increase in intracellular calcium influx. Akt and NF-κB are downstream effectors of PKC pathway in regulating flow-induced survivin expression. The increase in p-NF-κB expression is accompanied by an increase in survivin expression, which is critical for normal endothelial cell division and consequently vascular integrity. Modified from [152, 153] with permission.

for proper heart morphogenesis and development. Cilia mutants have been characterized by heart developmental defects such as ventricular and atrial septum defects, myocardial wall disorganization and thinning and double-outlet right ventricles due to mutations in *pkd1* or *pkd2*, the causative genes of ADPKD [93]. Moreover, knockout mice of cilia structural genes such as *ift88* and *kif3a* are also characterized by severe cardiac phenotypes such as hypoplasia of the endocardial cushions, reduction in ventricular trabeculation and enlarged pericardium [94]. This suggests that these cardiac phenotypes could be contributed mainly by ciliary dysfunction and that primary cardiac cilia are critical for normal heart development. More importantly, dysfunctional cilia are associated with congenital heart disease. Several ciliopathies such as nephronophthisis (NPHP), Meckel syndrome (MKS), Bardet-Biedl syndrome (BBS), and Joubert syndrome (JBTS) are usually associated with cardiac defects such as septum defects and aortic stenosis [95].

Cardiac development is controlled by complex regulatory networks of both inductive and inhibitory signals from both the heart as well as the surrounding tissue which coordinate different stages of heart development. An example is the Hedgehog (Hh) signaling pathway which regulates the L-R asymmetry in vertebrates and promotes the activation of several transcription factors involved in various cellular mechanisms during tissue homeostasis and development. It has been shown that primary cilia can regulate the Hh signaling pathway in several cell types by functioning as a unique compartment for the continuous turnover of Hh signaling components. As a result, defects in primary cilia assembly and consequently Hh signaling components turnover can lead to a variety of developmental pathologies including congenital heart defects [96]. Another example of a signaling network which plays a critical role during heart development is the TGFβ/Bone Morphogenic Protein (BMP) network. It has been recently shown that primary cilia can regulate the canonical TGFβ signaling network through the activation of transcription factors Smad2/3 [97]. Furthermore, it has been shown that the TGFβ ligand, TGFβ-1 can stimulate the differentiation of stem cells into cardiomyocytes and that *Tg737*$^{Orpk/Orpk}$ mouse embryonic fibroblasts are characterized by diminished TGFβ activity, suggesting a direct role of cardiac primary cilia in regulating TGFβ signaling during cardiomyogenesis. Taken together, these studies prompted us to speculate that primary cilia may function as signaling hubs facilitating the cross-talk between the different signaling networks that regulate heart development.

## Primary Endothelial Cilia and Vascular Architecture

The morphology and function of blood vessels are continuously altered in response to blood flow which is mainly detected by the vascular endothelium. An aneurysm

is a balloon-like bulge in the wall of blood vessels. Studies on both ADPKD patients and animal models indicate that polycystins are essential for normal blood vessel's structure and development [98]. Aneurysm formation is one of the deadliest vascular abnormalities in PKD patients. The occurrence of aneurysm represents a major risk factor for morbidity and mortality associated with PKD [99]. Mice with *Pkd* mutations develop hemorrhages and aneurysms [100]. Vascular aneurysms are associated with tissue remodeling due to abnormal proliferation of the endothelial cell layers in response to complex hemodynamic changes in fluid shear stress [101]. We recently demonstrated that aneurysm formation is more severe in *survivin, pkd1* and *Tg737* vascular-specific knockout (*PdgfβCre:Survivin* $^{flox/flox}$, *PdgfβCre:Pkd1* $^{flox/flox}$ and *PdgfβCre:Tg737* $^{flox/flox}$) mice than in wild type mice [102]. An abnormality of primary cilia structure (polaris) or function (polycystin-1) is associated with downregulation of survivin expression, which is associated with abnormal mitotic events in endothelial cells [103]. This is turn can lead to abnormal cytokinesis triggered by polyploidy formation and ultimately contributes to vascular aneurysm [102]. Thus, the inability of primary endothelial cilia to respond to fluid shear stress can contribute to the exacerbation of aneurysm formation in these mice models.

Protein Kinase C (PKC) and Akt are downstream signaling messengers of primary cilia. Studies on endothelial cells have verified that survivin expression following cilia activation is regulated by PKC, Akt and Nuclear Factor-κB (NF-κB). Akt is downstream of PKC and can regulate NF-κB, which is known to regulate survivin expression. Taken all the above together, we propose a Cilia-PKC-Akt-NF-κB pathway involved in survivin expression and cell division regulation [102, 104-107]. All in all, the inability of mechanosensory cilia to detect fluid shear stress is associated with down-regulation of survivin leading to abnormal cytokinesis triggered by polyploidy formation and finally contributing to vascular aneurysm (Fig. **2b**) [102].

## Primary Cilia in Corneal Endothelium

The corneal endothelium located beneath the anterior chamber of the eye is orchestrated into a two-dimensional layer of hexagonal cells and plays critical role in maintaining corneal transparency. The presence of primary cilia in the corneal endothelium has been demonstrated in various mammalian species [44]. In humans, corneal endothelial cilia protruding into the anterior chamber of the eye have similar structure to the "9+0" primary cilia, but their function remains somehow unclear, although evidence for an osmoregulatory or a chemosensory function exists. Moreover, it has been postulated that primary cilia of the mouse corneal endothelium play key role towards proper morphogenesis of the characteristic hexagonal shape or pattern of the corneal endothelium during development and disassemble following tissue homeostasis during adult stage pointing towards a role of corneal endothelial primary cilium in corneal endothelial development [108].

More importantly, a link between primary cilia and intraocular pressure regulation has been recently demonstrated through studying a rare X-linked congenital syndrome known as pediatric glaucoma or Lowe syndrome [109]. It has been previously shown that the *Oculocerebrorenal syndrome of Lowe* (*OCRL*) gene encoding an inositol polyphosphate 5- phosphatase, which is mutated in Lowe syndrome is involved in vesicle trafficking to the primary cilium [110]. More recently, a potential role of primary cilia in the trabecular meshwork which regulates intraocular pressure in the eye has been demonstrated. In this study, it was shown that primary cilia are important sensors of intraocular pressure changes and that in glaucoma, defective trabecular meshwork cells have shortened primary cilia due to increased intraocular pressure and fluid flow [109]. Consequently, this shortening is associated with increased expression of TNFα, TGFβ and GLI1 genes. Primary cilia response to increased intraocular pressure is mainly mediated through interaction between OCRL and transient receptor potential vanilloid 4 (TRPV4), a ciliary mechanosensory channel. Although these findings significantly advance the current understanding of how intraocular pressure is regulated, they provide further evidence and support for our proposed role of primary endothelial cilia as both blood pressure sensor and regulator.

## Primary Cilia and Vascular Smooth Muscle Cell Function

Vascular smooth muscle cells (VSMCs) localized in the medial layer of the arterial wall play critical roles in maintaining and regulating blood vessel tone, blood pressure and blood flow [111]. VSMCs express a unique group of proteins with specialized contractile functions and have very low proliferative profile during adult stages. The environment in which VSMCs reside is mainly composed of collagen and elastic fibers as extracellular matrix (ECM) components. Normally, VSMCs are not exposed directly to fluid shear stress simply because the inner lining endothelial cell layer provide the contacting surface with blood flow and consequently shields the VSMC layer. However, in cases of endothelial cell layer injury or denudation, VSMCs become directly exposed to fluid shear stress levels similar to the endothelial cell layer in intact vascular regions. It is critical to understand the mechanisms by which VSMCs sense and transduce the stimuli of shear stress into intracellular biochemical signals which allows better understanding of vascular disease. Studies reviewing the effects of fluid shear stress on VSMCs revealed the presence of shear specific mechanosensors such as cell membrane-related receptors, cell surface glycocalyx, ion channels and integrins as well as the presence of shear-specific secondary signaling messengers such as NO, $Ca^{+2}$ and MAPKs. These messengers in turn regulate the expression of shear-responsive genes which control various VSMCs responses such as proliferation, differentiation, apoptosis and migration.

A sensory role of primary cilia in VSMCs similar to its role in several other cellular systems has not been demonstrated until recently [112]. It has been shown that primary cilia are localized to VSMCs in a preferentially oriented pattern with respect to the artery axis as well as to the ECM and they house mechanosensory proteins that are responsive to ECM proteins and to cell-ECM interaction. These initial observations suggest a mechanochemical

sensory role of VSMCs primary cilia in the vasculature. In addition, primary cilia on the surface of VSMCs may act as fluid mechanosensors regulating intracellular $Ca^{+2}$ influx and VSMCs migration in response to fluid shear stress. Primary cilia and cell surface glycocalyx are both displaced in response to fluid flow and affect cell surface receptors as well as integrins. Both cell contraction and migration requires disassembly of integrin-mediated focal adhesion [113]. Therefore, it remains to be discovered if VSMCs primary cilia can sense interstitial flow and play a role in controlling VSMCs contraction and migration. In addition, VSMCs *in vivo* are exposed to other hemodynamic forces such as stretch, and pressure at the same time, which may act in concert to regulate mechanosensitive signaling pathways controlling vascular function and disease. In summary, it is safe to assume that if VSMCs' primary cilia function or structure is perturbed, it will contribute to vascular disease and hypertension.

## HYPERTENSION AND HUMAN ADPKD

ADPKD is an example of a ciliopathy or a genetic disorder resulting from the abnormal function and/or structure of primary cilia. Even with successful renal transplant or replacement therapy, patients with ADPKD eventually die due to cardiovascular complications, including hypertension, aneuryms, aortic root dilation, dissection, vascular ectasia, mitral valve prolapse and abnormal function of the microvascular bed [99, 114-116], heart failure and congestive heart disease [115-121]. Aside from the kidney phenotype, the frequencies of phenotypic manifestations in patients with ciliopathy include congestive heart failure/hypertension (78%), hepatic cysts (75%), diverticulosis (70%), ovarian cysts (40%), cardiac valve disorders (25%), inguinal hernias (15%) and intracranial aneurysms (10%) [107]. Our group is one of the first to report cilia function using mouse models and patient samples to explain cystic kidney phenotypes [2, 122] and to report cilia function in nitric oxide (NO) biosynthesis [5, 123]. Plasma concentration of the vasodilator nitric oxide has been found to be lowered in ADPKD patients compared to healthy volunteers [124]. This demonstrates a strong link between endothelial dysfunction and ADPKD. Another study reported similar results where the level of ADMA (asymmetric dimethylarginine), a marker of an inhibitor of nitric oxide synthase, was found to be elevated in PKD patients [125].

The correlation between hypertension and kidney volume occurs in the early childhood stages of ADPKD. The development of hypertension in ADPKD patients is associated with increased renal volume as well as increased left ventricular mass index. Similarly, it has also been suggested that high blood pressure can advance cyst growth [126, 127]. Interestingly, hypertension appears in children before they confront any significant reduction in glomerular filtration rate or development of ADPKD [128, 129]. Now that ADPKD is strongly correlated with dysfunction of ciliary proteins and/or abnormal cilia structure [2, 130, 131], this prompted us to propose that, endothelial dysfunction in ADPKD patients may be attributed to the inability of primary endothelial cilia to sense fluid-shear stress, or to an anomaly in any other downstream signaling mechanism. Taken together, this suggests that the pathogenesis of hypertension is a risk factor of endothelial primary cilia dysfunction at least partially independent from kidney function in ADPKD.

On the other hand, renal cyst enlargement in ADPKD adults has been ascribed to contribute in part to hypertension. Enlargement of renal cysts leads to the stimulation of both circulating as well as the intrarenal renin-angiotensin-aldosterone system (RAAS) system due to the compression of the cyst-adjacent parenchyma on the renal vasculature resulting in areas of renal ischemia and vascular changes [132, 133]. Activation of RAAS in PKD has been substantiated in both clinical studies [134] and murine models [135, 136]. Other components of the RAAS, including angiotensinogen, angiotensin converting enzyme (ACE), angiotensin II receptor and angiotensin II peptide have also been detected in cysts and dilated tubules in ADPKD kidneys [137]. In addition to hypertension, angiotensin contributes significantly to cyst growth and expansion, increased endothelin levels, fibrosis and increased sympathetic activity. Sympathetic activity can stimulate RAAS and angiotensin can stimulate sympathetic nervous system as well [138, 139]. Moreover, activation of the RAAS has been found in normotensive and hypertensive PKD patients, regardless of their blood pressure and renal function [140]. Not surprisingly, the high levels of circulating angiotensin II in PKD patients have been shown to contribute to the development of vascular hypertrophy, which is further implicated in vascular remodeling [141]. The effect of blockage of the renin-angiotensin-aldosterone system with Angiotensin Converting Enzyme inhibitors (ACE-I) and angiotensin receptor antagonists on renal volume and kidney function is discussed in the next section. A study involving eleven kidney transplant cases of hypertensive PKD patients accounted an improved blood pressure in only six patients [142]. Another report demonstrated that PKD patients with hypertension continue to show cardiovascular complications after renal transplantation [143]. These studies clearly suggest that, though kidney transplantation is beneficial for improving some cardiovascular issues, it is not sufficient to control hypertension in PKD patients. Whether hypertension contributes to diminished renal function or deteriorated kidney function exacerbates hypertension is still a debated topic that warrants further discussion and there is a wide scope of more research to clearly understand the cause and effect phenomenon undergoing in this case.

## AVAILABLE AND ONGOING TREATMENT OPTIONS FOR CARDIOVASCULAR DISEASES IN ADPKD

A recent review involving 1877 ADPKD patients' records showed that the use of antihypertensive therapy in ADPKD patients have been increased from 32% in 1991 to 62% in 2008. A similar increase in the use of RAAS inhibitors was also reported (from 7 % to 42 %) [144]. But given that cardiovascular complications are the most

common cause of death in ADPKD patients, it is justified that researches are going on to a greater extent for innovation of novel therapeutics.

Alike regular hypertensive patients, reduced salt and caffeine intake, smoking cessation, regular exercise, and optimal water consumption have been suggested and found beneficial in hypertensive PKD patients [145, 146]. Pathophysiology of hypertension in this disease suggests the usefulness of ACE inhibitors. ACE-I enalapril is found to control blood pressure but it has a consistent antiproteinuric effect [133]. In a different study, 3% of patients displayed a reversible increase in serum creatinine after initiation of ACE-I use [147, 148]. So, ACE-Is should be used cautiously in ADPKD patients. The RAAS based pathophysiology of hypertension foretells the effectiveness of Angiotensin Receptor Blockers (ARBs). A randomized trial compared candesartan (ARB) with amlodipine (calcium channel blocker) and both were found equally effective in controlling blood pressure in PKD patients [149]. However, there is no other study exhibiting similar outcome and should be explored further. Dual blockade of RAAS with ACE-Is and ARBs has also been suggested and being tested in two clinical trials [150]. The most recent release of the results of the HALT study among 558 hypertensive participants with ADPKD revealed that the combination of lisinopril, an ACE-I and telmisartan, an ARB did not significantly alter the rate of increase in total kidney volume in the early stages of ADPKD. However, they indicated that aggressive blood-pressure control might slow kidney cyst growth and reduce left-ventricular-mass index compared to normal blood pressure control [151]. Beta-blockers (metoprolol, atenolol) and calcium channel blockers (amlodipine) are found to be effective in controlling hypertension in PKD patients. Though metoprolol did not alter glomerular filtration rate (GFR) or urinary albumin excretion, effects of atenolol on kidney function is still unknown. Calcium channel blockers are not recommended for use as a first line therapy in PKD patients, as this may cause a significant reduction in GFR. Theoretically, diuretics are recommended in PKD patients only as an additional therapy with ACE-Is as diuretics activate RAAS. Moreover, patients treated with diuretics showed increased urinary excretion of proteins [150].

We and others have independently studied abnormality in primary cilia in the vascular and renal systems in PKD. We hypothesized that cilia in the vascular system play important roles in regulating blood pressure and vascular integrity through the expression of mechano- or chemosensory receptors. Supporting our hypothesis is that patients with abnormal cilia function (PKD) also show a greater incidence of hypertension and other cardiovascular problems. In theory, drugs that interfere or alter cilia sensory function could prevent or delay this high blood pressure increase. This idea is based on our most recent chemical-screening studies showing that dopamine receptor is localized in endothelial cilia and that this ciliary receptor has dual chemo and mechano-sensing roles in endothelial cells. We showed that "activated" dopamine receptor-type 5 in the cilia could switch its chemosensing function to mechanoreceptor, while "non-activated" receptor has a primary function as a chemoreceptor. Activation of ciliary dopamine receptor could bypass mechano-ciliary polycystin complex through a mechanism that increases the cilia length. The extension of cilia probably serves to increase the sensitivity of these sensory organelles to fluid flow, in which dopamine receptor-type 5 acts as the mechanoreceptor.

## CONCLUSION

As more evidence is presented to support the direct involvement of primary cilia in the cardiovascular system, this necessitates large-scale screening studies for drugs to investigate whether targeting primary cilia function or structure towards hypertension results in better outcomes.

## CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

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# Graphical Abstract

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## Vascular Endothelial Primary Cilia: Mechanosensation and Hypertension

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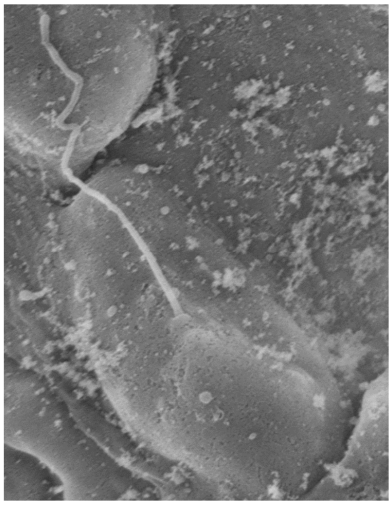

**Primary cilia are mechanosensory organelles extending from the cell surface to sense cues in the extracellular environment and transduce signals into the cell producing a response that regulate a physiological phenomenon like blood pressure.**